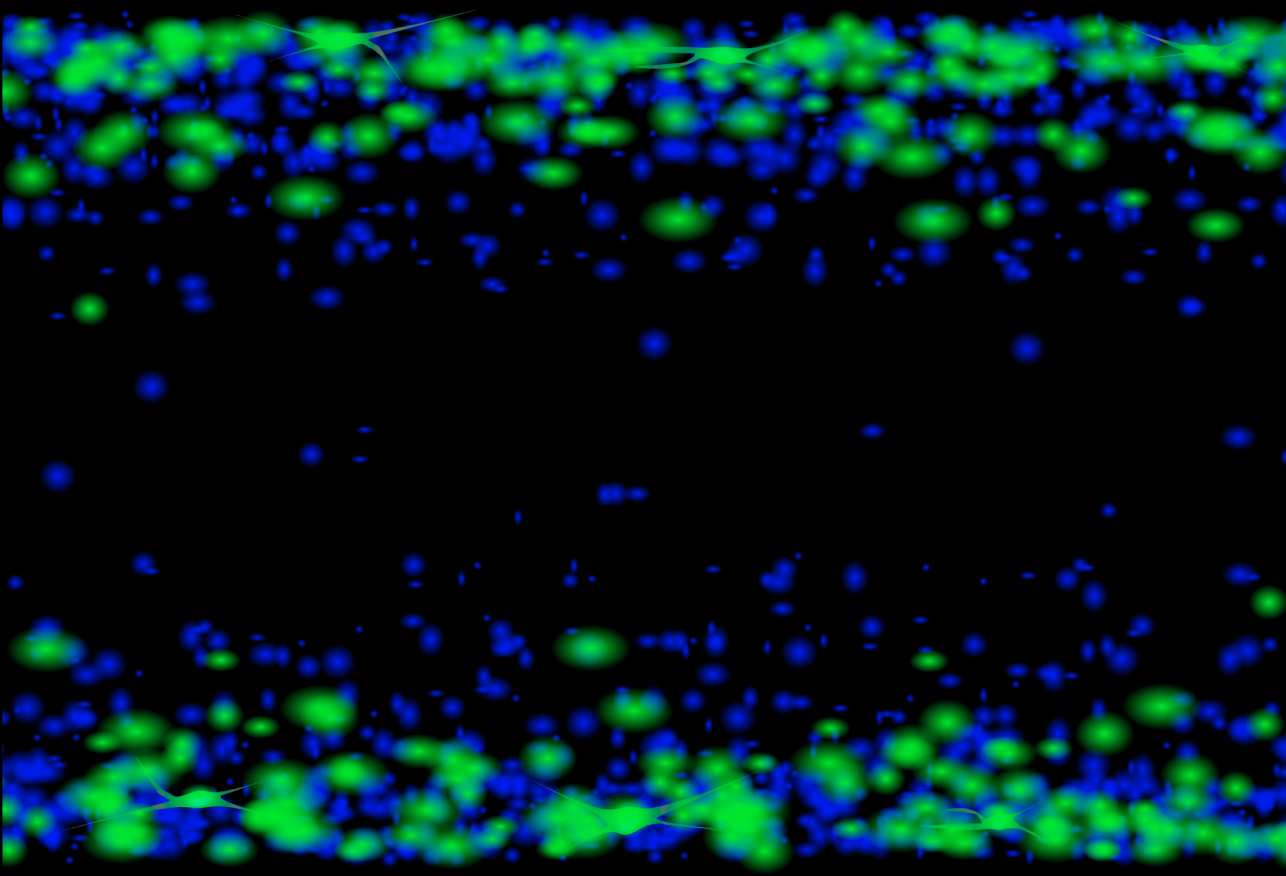
Disease-regulated gene therapy for arthritic diseases

From experimental arthritis to human *in vitro* models



Mathijs G.A. Broeren

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Martinus Gerardus Antonius Broeren

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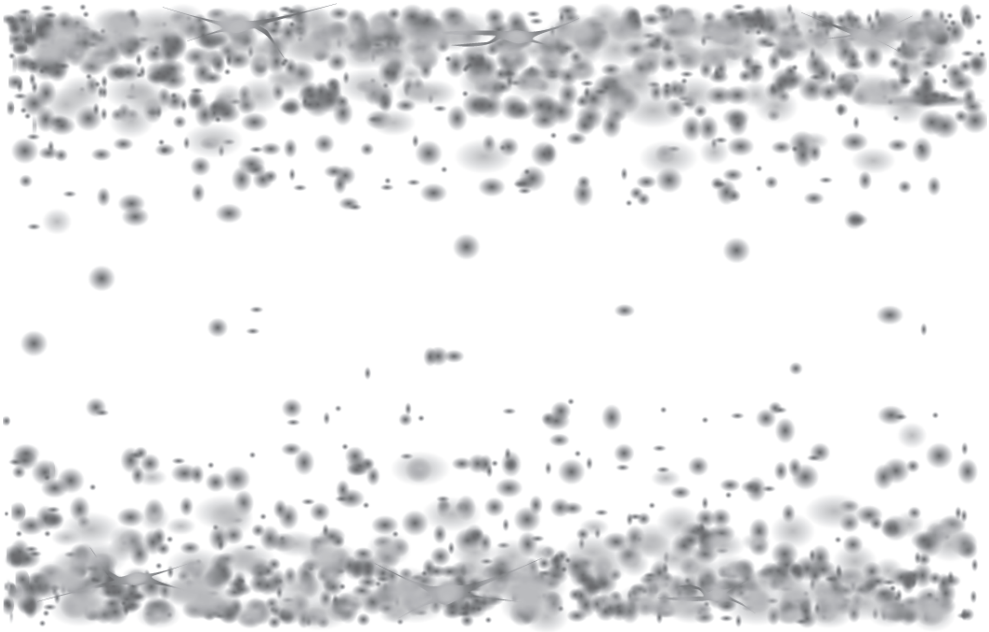

Chapter 1

General introduction

Mathijs G. A. Broeren

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a debilitating disease affecting about 1% of the world population. It is an autoimmune disease that leads to inflammation and eventually destruction of joints **(Fig. 1).**The exact etiology is still not clear and seems to be multi-factorial with risk factors including a genetic predisposition, smoking, obesity and a female gender (Symmons et al. 1997, van der Helm-van Mil et al. 2006). At diagnosis, 50-70% of the patients are positive for antibodies directed against citrullinated proteins (ACPA, anti-CCP) or IgG (rheumatoid factor, RF) in the serum (Smolen et al. 2016). Several proteins that occur in the joint can be citrullinated, which is thought to provoke the inflammatory response if encountered by autoantibodies. Many different cell types are involved that orchestrate the inflammation by producing many different pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6). The production of cytokines triggers the production of matrix-degrading enzymes and attracts even more immune cells, ultimately leading to joint malformations and irreversible damage. As of yet, RA cannot be cured, but the inflammation is efficiently suppressed by disease-modifying anti-rheumatic drugs (DMARDs). These include methotrexate (MTX) and biological drugs (biologics), which are recombinant proteins that are injected at regular intervals, usually weekly. Although the biologics slow down the progression of RA, the continuous systemic exposure can lead to opportunistic infections and anti-drug antibodies (Aubin et al. 2013)

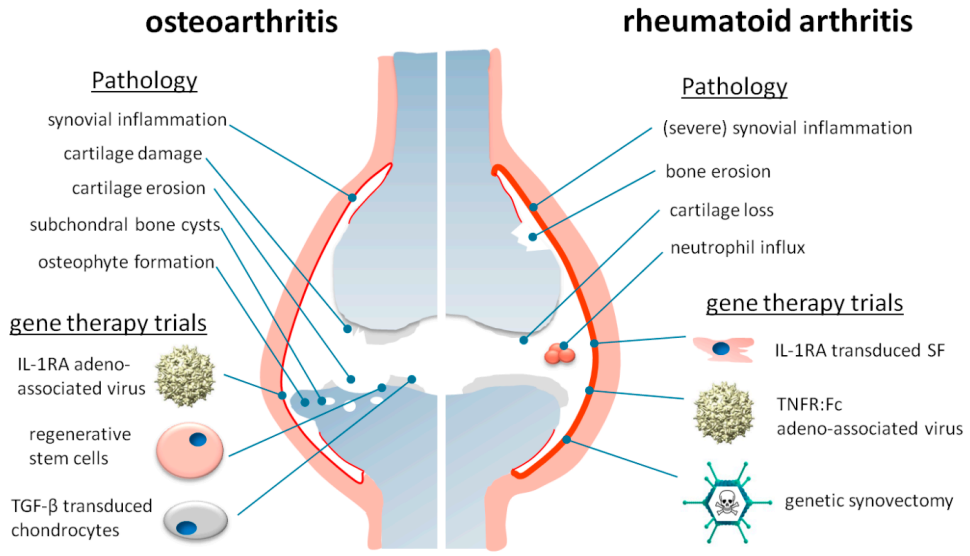

Figure 1: Comparison of a knee joint with osteoarthritis and rheumatoid arthritis, depicting the pathology and the performed gene therapy trials. The femur and tibia are shown covered by a protective layer of cartilage. In between is the synovial cavity which is filled with synovial fluid containing nutrients and lubricating proteins. The composition of the synovial fluid is maintained by the cells in the synovial lining surrounding the synovial cavity. In osteoarthritis, synovial inflammation is observed in a subset of patients. In addition, cartilage pathology is observed and bone spurs (osteophytes) develop along the edges of the bone. Rheumatoid arthritis is associated with more severe inflammation, which is also associated with neutrophil influx. Pannus tissue forms from the synovium which causes bone erosion. For osteoarthritis, several gene therapy approaches are under develompent including an adeno-associated virus for expression of IL-1RA which has been successfully tested in equine osteoarthritis. Regenerative stem cells are considered cell therapy, but they may be transduced with disease-modifying viral vectors prior to administration (eg. MSC-Atsttrin). The TGF-β transduced allogeneic chondrocytes are currently in a Phase II clinical trial. In contrast, gene therapy trials in rheumatoid arthritis have focused exclusively on inhibiting the inflammation by targeting IL-1 signaling, TNFα signaling or by depleting immune cells in the joint. IL-1RA: interleukin-1 receptor antagonist, SF: synovial fibroblast, TGF-β: transforming growth factor beta, TNFR:Fc: fusion protein of the tumor necrosis factor receptor and immunoglobulin Fc.

Osteoarthritis

A second rheumatic disease that is a major focus of research is osteoarthritis (OA), which is the most common joint disease. OA is not an autoimmune disease, but similar to RA, OA affects the joints and the exact cause is unknown. Risk factors for OA include age, obesity and previous joint trauma (Blagojevic et al. 2010). However, unlike for RA, no disease-modifying drugs are available for OA and affected joints are often ultimately surgically replaced. For many years, people have viewed OA as mechanical wear-and-tear of the cartilage, but in recent views OA is considered an active disease affecting the whole joint (Malemud 2015) **(Fig. 1)**. Inflammation is observed in up to half of the OA patients and is hypothesized to be involved in the pathogenesis (Blom et al. 2007, Wojdasiewicz et al. 2014, de Munter et al. 2016).

Because no disease-modifying treatment is available yet, there is a high demand for an effective therapy to treat OA. Despite the association of inflammation with OA, the biological therapies used to treat RA have insufficient effect in OA (Chevalier et al. 2013). An explanation for the lack of effects from these systemic treatments is that OA is considered a local process and delivery of protein-based molecules to the joint is challenging (Chevalier 2010). The penetration of systemically administered protein-based drugs into the joint is low and repetitive intra-articular injections cause high discomfort for the patient (Wallis et al. 1987). Local gene

therapy with viral vectors might provide long-term expression of a protein drug in the joint, while preventing continuous exposure of the body to immunosuppressive agents.

On the origin of gene therapy

Ever since the identification of DNA as the carrier of inheritable information and the capacity of viruses to transfer genetic material into cells in the 1960s, there have been speculations to make use of 'gene therapy' to correct molecular defects (Tatum 1966). After the development of additional molecular biological techniques, the first approved and successful clinical trial was started in 1990 when 2 patients suffering from adenosine deaminase deficiency – severe combined immunodeficiency (ADA-SCID) were treated with a retrovirus encoding adenosine deaminase (Blaese et al. 1995). In patients suffering from ADA-SCID, dATP builds up in all cells, which results in loss of T- and B-cells leading to opportunistic infections. In the trial, one of the two patients showed structural improvement in adenosine deaminase activity and an increase in T-cell numbers over a course of 4 years. The first publication on successful gene therapy however, described the *ex vivo* retroviral transfer of the LDL-receptor to autologous hepatocytes for the treatment of familial hypercholesterolemia (FH) (Grossman et al. 1994). One patient was included in this study, with positive effects on the LDL/HDL ratio for at least 18 months. Importantly, no genotoxicity was observed in these studies, which had been a major concern for the integrating retroviral vectors. Recently, several additional successful gene therapy clinical trials have been performed, described in Text Box 1.

Text Box 1: recent successes of gene therapy

In recent years, the field of gene therapy had several successful developments. The first gene therapy approved for the European market was alipogene tiparvovec (trade name Glybera) in 2012, which was developed by a Dutch company for the treatment of LPL (lipoprotein lipase) deficiency (Yla-Herttuala 2012). Recently, two other gene therapies have been approved: The oncolytic T-VEC virus (Fong 2015) and the gene therapy for ADA-SCID described above (Yla-Herttuala 2016). Additional successful clinical trials have recently been performed with gene therapy for Wiskott-Aldrich syndrome (Hacein-Bey Abina et al. 2015), the progressive retinal degenerative disease Leber's congenital amaurosis (Bainbridge et al. 2015), neovascular age-related macular degeneration (Rakoczy et al. 2015) and a gene editing approach for the treatment of HIV (Tebas et al. 2014). Interestingly, these studies made use of adeno-associated viruses (AAVs), lentiviruses and retroviruses, thus providing extensive safety and expression kinetics data for different viral vectors for over 3 years. No severe pathogenic mutagenesis has been observed in any of these trials and individual integration sites were not associated with increased cell proliferation. Positive safety aspects of these vectors and extensive knowledge of their behavior are essential for the application of gene therapy for non-lethal diseases, like RA and OA.

Gene therapy vectors

A description of the term 'gene therapy' is provided in Text Box 2. Roughly, gene therapy agents can be divided into several categories, based on vector properties. The most common distinction made is between viral and non-viral vectors. The non-viral vectors are a diffuse group of recombinant gene (transgene) delivery strategies that includes all vectors with the exception of the viral vectors. In general, non-viral vectors are considered less immunogenic compared to viral vectors and can be produced in high quantities to obtain high vector to target cell ratios (Chevalier et al. 2009). High relative vector quantities are required for non-viral transfections, because they lack the evolved sophisticated virus strategies for transfection efficiency, stability and transcriptional activity. Several chemical, physiological and biological strategies can improve the efficiency and stimulate uptake of plasmids by specific cells (Nayerossadat et al. 2012). For example, plasmids can be incorporated in liposomes, which are lipid vesicles that can be coated with specific homing antibodies (Xing et al. 2016).

Text Box 2: The definition of gene therapy

The exact definition of gene therapy has changed over time due to new ideas for additional applications (from only correcting single monoallelic defects in the initial definitions to the restoration of the cytokine balance as suggested for RA gene therapy) and the development of new techniques (eg. gene editing). The American Society for Gene and Cell Therapy (ASGCT) currently defines gene therapy as follows: 'Gene Therapy can be defined as the use of genetic material (usually deoxyribonucleic acid - DNA) to manipulate a patient's cells for the treatment of an inherited or acquired disease'. The U.S. Food and Drug Administration (FDA) currently maintains a long definition including the core sentence: 'Gene therapy is a medical intervention based on modification of the genetic material of living cells'. Gene therapy is often confused with cell therapy. Somatic cell therapy is defined by the FDA as 'the administration to humans of autologous, allogeneic, or xenogeneic living non-germline cells, other than transfusable blood products, for therapeutic, diagnostic, or preventive purposes.' Gene therapy and somatic cell therapy can be combined if cells are first transduced with a vector and subsequently administered to a patient. The careful correct terminology for all currently approved gene therapy trials is 'somatic gene therapy' to distinguish from germline gene therapy. If a viral vector integrates in germline cells, all future generations will carry the modification of which the consequences cannot be predicted. The risk of germline mutations in somatic gene therapy is therefore a constant concern. In several countries, including the Netherlands, germline gene therapy is forbidden due to technical and ethical considerations. The US currently has no federal legislation, but the FDA does not currently approve clinical trials based on germline gene therapy (Ishii 2015).

Viral vectors make use of the efficient and sophisticated properties evolved in naturally occurring viruses to obtain expression of the desired transgene. Naturally occurring viruses contain DNA or RNA that encodes the blueprint of the virus. After

a target cell is transduced, the cell will replicate the viral genome and express the required proteins to assemble new virus particles. The newly produced virus particles can infect new cells to propagate the viral infection. In viral vectors used for gene therapy, the virus genome is partly replaced by a therapeutic gene. The viral vector particle can transduce a target cell once and start production of the therapeutic gene. Because the viral vector does not encode the complete viral genome, no viral proteins are expressed to form new virus particles and the vector can therefore not replicate. The most commonly used viral vectors can be divided into integrating vectors (eg. retrovirus, lentivirus) and mostly episomal vectors (eg. adenovirus, AAV). Integrating viruses integrate in the host genome and provide long-term expression of a transgene. When a transduced cell proliferates, it will also carry over the integrated vector. The major down-side of the integrating vectors is the risk of disrupting tumor-suppressor genes or activating proto-oncogenes (see Text Box 3). This risk is reduced when using episomal vectors. However, episomal vectors are degraded in time by the transduced cells and the vectors are divided over the daughter cells during replication. In general, different viral vectors can transduce different sets of cell types (tropism) and have a different transgene capacity (Bouard et al. 2009). Certain viruses, including retroviruses can only transduce dividing cells and viral vectors are to a different extend hampered by pre-existing and vector-induced neutralizing antibodies (Calcedo et al. 2009). Several reviews have been written on the specific characteristics and limitations of the different viral vectors (Gardlik et al. 2005).

In this thesis, we use lentiviral (LV) and adenoviral (AdV) vectors. LVs are derived from the human immunodeficiency virus (HIV) and can be integrated in the genome of non-dividing cells, which is an advantage compared to the integrating retroviral vectors. (Matrai et al. 2010). LVs have a high transgene capacity of ~10 kb, although even larger transgenes can be incorporated with a decreased yield (Kumar et al. 2001). We use the self-inactiving (SIN) LV vector system in which the genes required to create a replication-deficient virus are dispersed over 4 expression vectors that are co-transfected in 293T cells. The resulting LV does not contain RNA coding for viral genes and contains a partly defective long terminal repeat (LTR) that cannot induce the expression of nearby (proto-onco)genes. The expression of transgenes in the SIN vector is instead regulated by a newly inserted promoter. The tropism of the original HIV lentivirus is restricted to $CD4^+$ cells. By replacing the original gp120 envelope protein with vesicular stomatitis virus G (VSV-G) protein, the virus tropism is greatly increased to many different cell types (Cronin et al. 2005). The low-density

lipoprotein (LDL) receptor has recently been identified as the main receptor for the VSV-G, explaining the broad tropism of VSV-G pseudotyped LVs (Finkelshtein et al. 2013). In general, we apply LVs in the experiments where long-term expression was desired. Similar to the LV, AdV has a broad tropism, can transduce non-dividing cells and has a high transgene capacity (5.1 kb, which can be increased to up to 37 kb if additional viral genes can be replaced) (Danthinne and Imperiale 2000, McConnell and Imperiale 2004). In contrast to the LV however, the AdV does not incorporate into the host genome, providing a more transient expression and we apply the AdV if particularly high and rapid transgene expression is desired.

The AdV we use is deficient for the Early-1 (E1) gene, which is expressed by the N52E6 amniotic cell line that is used for AdV production (Schiedner et al. 2000). The promoter sequence and the poly-A sequence surrounding the adenoviral E1 gene in the N52E6 cells have been replaced by non-adenoviral substitutes. This prevents homologous recombination of the E1 gene into the produced AdVs, thus preventing the creation of replication-competent AdVs (RCAs) and improving the recombinant AdV safety.

Text Box 3: gene therapy setbacks

After the hopeful results of the first gene therapy clinical trials, several patients suffered severe adverse side effects in subsequent trials. A major setback occurred in 1999, when a patient died in a trial with gene therapy for patients suffering from Ornithine transcarbamylase deficiency (OTCD) 96h after receiving the highest AdV dose of the study (Raper et al. 2002). The death was attributed to the administered AdV, which resulted in a very strong immune response and multiple organ failure. Although the subjects receiving a lower dose AdV also showed an inflammatory reaction to the vector, it remains unclear what caused the exaggerated immune response in the deceased patient (Raper et al. 2003). A milder immune response was observed in the second patient receiving the highest vector dose, but the clinical trial was aborted.

In another trial for X-linked SCID (SCID-X1) with *ex vivo* retroviral transduction of bone-marrow-derived $CD34^+$ cells with the IL-2 receptor γ gene, 4/10 patients developed leukemia of which one patient eventually died (Hacein-Bey-Abina et al. 2008). The leukemia might have been caused by an unfortunate combination of the use of a retrovirus with an LTR enhancer, integration near the *LMO2* proto-oncogene and expression of a pro-survival transgene in patients with a severe immune deficiency. These severe side effects overshadowed the fact that most treated children are still alive today, owing to the gene therapy.

Gene therapy in Rheumatoid arthritis

Intra-articular gene therapy can be a useful apporach to provide long-term expression of potentially therapeutic proteins in the joint without administering weekly i.a. injections. The first gene therapy for rheumatic diseases was proposed in 1992 (Bandara et al. 1992). At the time, the first protein-based treatments were tested in arthritis models. The first treatments mainly targeted IL-1 and systemic

injections with recombinant IL-1 receptor antagonist (IL-1RA) could reduce IL-1-induced synovitis and proteoglycan depletion (Henderson et al. 1991). Bandara and colleagues proposed that local gene therapy could overcome the challenges of physiological instability of therapeutic proteins and poor penetration of the joint after systemic injection. The local treatment could be ideal to treat oligoarthritis or specific joints refractory to mediation. The route of administration could be either by direct injection of the virus or by injecting *ex vivo* transduced autologous joint cells.

The promising results with IL-1RA in animal models resulted in the approval of the first of few clinical trials for RA in 1995. Of 9 patients scheduled for joint replacement surgery of multiple metacarpophalangeal (MCP) joints, one joint was replaced earlier and the cells retrieved from the joint were transduced with a retrovirus encoding IL-1RA **(Fig. 1)**. The transduced cells were injected in the second joint and after 1 week, the joints were removed and analyzed. No adverse events occurred and IL-1RA was successfully expressed. The production of both IL-6 and prostaglandin E2 were reduced by the treatment. A second clinical trial with a similar approach and a 4-week follow-up was initiated. Two patients were treated and no adverse events were reported (Wehling et al. 2009). The injection of transduced cells reduced the pain score in the joints compared to injection of non-transduced cells. However, after the inclusion of these 2 patients the leukemia in the SCID-X1 trial occurred (Text Box 3), which used a similar retroviral vector and the clinical trial was aborted. Despite these promising results no additional trials have been initiated with this therapy, possibly in part because of the developing insights that the efficacy of IL-1Ra biologicals is inferior to anti-TNFα biologicals (Kalliolias and Liossis 2008). It has been estimated that a 100-fold molar excess of IL-1RA is required for successful *in vivo* suppression of IL-1 activity (Arend 1993).

After the development of biological drugs for the treatment of RA, a second approach involving gene therapy was tested in a clinical trial. A recombinant adeno-associated virus serotype 2 (rAAV2) was used to express the cDNA of the extracellular portion of the TNFα receptor fused to the Fc portion of IgG (TNFR:Fc, tgAAC94), a transgene similar to the etanercept biological. After the successful treatment of experimental arthritis and positive safety data in non-human primates, 15 patients received a single intra-articular injection and no severe side effects relating to the viral vector were observed (Mease et al. 2009). Subsequently, the efficacy was tested in a phase 1/2 study on 127 patients **(Fig. 1)**. Two virus injections were planned, but several patients did not receive the second injection after one of the patients died (Mease et al. 2010). Only marginal improvements were observed compared to the placebo-injected group. The death was attributed to an opportunistic

histoplasmosis. It could not be determined to what extent the immune suppression by the viral vector contributed compared to other immunosuppressive medication the patient received simultaneously (Frank et al. 2009). To our knowledge, the IL-1RA retrovirus and the TNFR:Fc AAV are the only RA gene therapy clinical trials for which the results have been published. One last notable gene therapy approach for RA gene therapy is by depleting the inflammatory cells in the joint using 'genetic synovectomy' **(Fig. 1)**. Using an intra-articularly injected adenovirus, the synovial cells can express the herpes simplex thymidine kinase (tk) gene. Cells expressing this gene can metabolize the drug Ganciclovir into a deoxyguanosine triphosphate (dGTP) analogue. The analogue is incorporated in the genome, but cannot be replicated by DNA polymerase causing apoptosis. This system has been successfully used to treat collagen-induced arthritis in monkeys by killing >90% of the synoviocytes (Goossens et al. 1999), although long-term effects on joint damage were not assessed in this study. A gene therapy clinical trial based on this approach has been started, but was stopped after inclusion of 1 patient, because of problems with patient recruitment (Evans 2005).

Gene therapy in osteoarthritis

In addition to an application in RA described above, IL-1RA therapy has been explored for efficacy in OA. In mouse models of OA, most successful treatments have been directed at inhibiting matrix-degrading enzymes, or by stimulating anabolic processes using various growth factors or regenerative (stem) cells **(Fig. 1)**. These approaches have been extensively reviewed by Goldring and Berenbaum (Goldring and Berenbaum 2015). One important target for OA gene therapy is IL-1β, which has been implicated to have a role in the pathogenesis of OA by maintaining a low-grade inflammation (Wojdasiewicz et al. 2014). IL-1β is upregulated in the synovial fluid, synovial membrane and in articular cartilage of OA patients and injections of recombinant IL-1β in mouse joints leads to cartilage proteoglycan depletion and increased expression of matrix-degrading enzymes. Repeated intra-articular injections of IL-1RA in a dog model of osteoarthritis resulted in reduced osteophyte formation and reduced cartilage damage (Caron et al. 1996). However, a single intra-articular injection of recombinant human IL-1RA (Anakinra) showed no therapeutic effects in a multi-center clinical trial (Chevalier et al. 2009). The half-life of Anakinra was determined to be only 4h, hence the potential benefit of a gene therapy approach to extend the exposure time to IL-1RA. Intra-articular gene therapy with an adenovirus coding for IL-1RA could reduce pain and cartilage damage in a horse model for osteoarthritis (Frisbie et al. 2002) **(Fig. 1)**. More recently, it has

been shown that horse joints transduced with a self-complementary AAV2 (scAAV2) vector can provide intra-articular expression of IL-1RA for up to 8 months (Goodrich et al. 2015). Several other studies have shown promising results with IL-1RA gene therapy for OA and in a recent study, the safety profile of an IL-1RA-expressing rAAV2.5 vector has been determined in a the rat mono-iodoacetate (MIA) OA model (Wang et al. 2016). No adverse effects were noted and the treatment could reduce OA features until 180 days after induction of the model, but beneficial effects were gone after 1 year. Although AAV vectors are considered relatively safe vectors, also in this study a dose-dependent formation of neutralizing antibodies against the AAV capsid was measured. This can potentially reduce the effects of subsequent vector injections and might require switching to a different AAV serotype. Of note is the importance to use the correct animal model for studying the effects of IL-1-related therapies, as it has recently been shown that the common collagenase-induced OA mouse model has comparable damage in IL-1α/IL1-1β knockout mice compared to wild-type mice, despite the clear presence of inflammation (van Dalen et al. 2016).

TGF-β as transgene for OA

Several other transgenes have been evaluated in single animal studies that could reduce experimental OA. However, the most progress toward human application of OA gene therapy has been made with an approach based on the application of chondrocytes stably transduced with a transforming growth factor beta 1 (TGF-β1) expressing vector **(Fig. 1)**. Interestingly, a disease-responsive metallothionein promoter was used to prevent excess exposure to TGF-β. A phase I clinical trial in 12 OA patients was initiated with a cell mixture designated TissueGene-C, consisting of a 3:1 ratio of non-transduced allogeneic chondrocytes and TGF-β transgenic chondrocytes irradiated with 15 Gy irradiation for safety (Ha et al. 2012). Some fluctuating clinical benefit could be observed for up to 12 months and some cartilage regeneration was observed on MRI. A phase IIa clinical trial was initiated in 27 patients with similar improvements after 12 and 24 weeks compared to baseline (Ha et al. 2015). Unfortunately, no control group was included in this study. Therefore, it is not certain which components of the TissueGene-C are required for the therapeutic benefit or if other treatment factors are of therapeutic influence (eg. the aspiration of all joint fluid before administration of the chondrocytes, or the reduced loading of the joints for 7 weeks). In a separate Phase II clinical trial, 57 patients were included and the high-dose TissueGene-C was compared to saline placebo (Lee et al. 2015). At 24 weeks, the clinical benefit of the TissueGene-C treated group was significantly higher compared to the placebo control group.

Although cartilage regeneration still remains to be determined in these phase II clinical trials, the clinical benefit was significant using different tests. Based on experiments from our department, caution should be exercised when applying TGF-β as a transgene, because adenoviral overexpression of TGF-β in mice resulted in osteophyte formation (Blaney Davidson et al. 2007). Moreover, a shift in TGF-β receptor analysis is observed during aging leading to a relative decrease in anabolic Smad2/3 signaling compared to detrimental Smad1/5 signaling (Blaney Davidson et al. 2009). This might negatively influence the therapeutic effects of TGF-β and was not investigated in the trials. The clinical trials using TissueGene-C are so far the only published gene therapy trials for OA.

Despite the several clinical trials with optimistic results for arthritic diseases described above, there is no gene therapy available yet for RA or OA patients in clinical practice. For RA, most gene therapeutic approaches are based on existing biologics that show limited health risks when delivered systemically as protein drugs. For OA, no biologics are available and less is known about the pathophysiology than for RA making a targeted strategy more challenging. In this thesis we aim to develop and test sophisticated viral vectors that can add to the current spectrum of anti-arthritic viral vectors by improving natural feedback mechanisms based on interleukin-10 (IL-10) and by providing transcriptional targeting using disease-inducible promoters. This approach can aid in the drug dose fine-tuning, which is currently an important topic for arthritis healthcare and will be further discussed in the 'inducible arthritis gene therapy' section of this introduction.

IL-10 therapy for RA

A major emphasis in this thesis is on gene therapy with IL-10. IL-10 is a powerful broad-spectrum anti-inflammatory cytokine, which can inhibit the production and signaling of pro-inflammatory cytokines, like IL-1 and TNFα (de Waal Malefyt et al. 1991, Fiorentino et al. 1991) and reduce antigen presentation (Frei et al. 1994). In contrast to the currently available biologics, IL-10 also occurs naturally in cells and is involved in the negative feedback of the inflammatory response. IL-10 can induce the expression of IL-1RA, soluble TNFα receptors and suppressor of cytokine signaling-3 (SOCS3) (Cassatella et al. 1994, Hart et al. 1996, Cassatella et al. 1999). In the first *in vivo* experiments with arthritis models, daily i.a. injections of recombinant IL-10 were found to inhibit collagen-induced arthritis (CIA) (Walmsley et al. 1996). The first application of IL-10 for the treatment of inflammatory diseases in humans was by daily injections of IL-10 for 7 days to treat Crohn's disease. 50%

of the patients showed clinical improvement after 3 weeks, compared to 23% of the placebo-treated group (van Deventer et al. 1997). A major challenge for therapy with recombinant IL-10 that became clear from these studies, is its short half-life of 1.1-2.6 hours. Patients with inflammatory diseases are prone to develop anemic conditions, which was observed after 4 weeks of daily IL-10 injections in patients with inflammatory bowel disease (Tilg et al. 2002). Several other side effects that can potentially be induced by continuous exposure to high levels of IL-10 are the increased expression of interferon gamma (IFN-γ) (Tilg et al. 2002) and increased Fc gamma-receptor expression in monocytes (Tilg et al. 2002). Disease-inducible gene therapy could be a suitable approach to provide long-lasting IL-10 expression, while minimizing the systemic off-target exposure to reduce these side effects.

IL-10 therapy for OA

IL-10 has also been a successful transgene in the treatment of animal models for OA. Rabbit synoviocytes transduced *ex vivo* with a retrovirus coding for IL-10 were injected in the knee joint and could reduce cartilage damage in a surgical model of OA (Zhang et al. 2004). IL-10 could also reduce blood-induced cartilage damage in human cartilage explants (van Meegeren et al. 2012). In addition to suppression of inflammation, IL-10 can inhibit the production of matrix-degrading enzymes (Behrendt et al. 2016). Several other studies have provided indirect evidence for an association of IL-10 with OA. For example, low innate production of IL-10 by blood cells after stimulation *ex vivo* is associated with an increased risk of OA (Riyazi et al. 2005). In another study, it has been concluded by systematic review that physical exercise has positive effects on pain and disability in knee OA (Roddy et al. 2005). These effects might be mediated by IL-10, which was found to be upregulated in the synovial fluid after exercise (Helmark et al. 2010). In addition to the inhibition of inflammation and matrix-degrading enzymes, IL-10 has chondroprotective and anabolic effects on cartilage by stimulating chondrocyte proliferation, stimulating the production of extracellular matrix components and reducing chondrocyte apoptosis (Jung et al. 2013, Wojdasiewicz et al. 2014). Despite the clear association of IL-10 with osteoarthritis, only a small amount of studies have tested IL-10 as a therapeutic option for OA and additional research is required to determine if IL-10 can be successful as transgene in OA.

Inducible arthritis gene therapy

For the use of biologics in RA, the earliest strategy was a dose escalation and nowadays a ‘hit hard’ approach is advocated followed by dose de-escalation.

Both approaches aim to find the optimal drug dose for each individual patient (den Broeder et al. 2010). The earliest gene therapy experiments focused on long-term high expression of transgenes in the joint. Dose escalations were tested in these patients, but only for safety assessment. We recognize that in addition to providing local expression, gene therapy vectors can also be designed to provide more specific targeting compared to systemic drug injections to further minimize off-target exposure. One approach is by isolating specific cell types for *ex vivo* transduction or by using viral vectors with a specific tropism. This has been extensively reviewed (van de Loo et al. 2006). A second strategy is by using specific promoters to regulate the expression of transgenes. These can include cell-specific promoters for cell-specific targeting, drug-inducible promoters, or promoters that are activated under specific disease circumstances, like inflammation. The focus of regulated gene therapy in this thesis is on disease-inducible promoters. These promoters are of particular interest for RA gene therapy, because many patients show an intermittent disease course (van der Helm-van Mil 2014).

Two major approaches have been explored to obtain disease-responsive promoters: 1 copying the promoter region of genes activated during disease and 2 generating artificial inducible promoters by combining a transcription start sequence with transcription factor binding sites. The first promoters from endogenous genes tested *in vivo* for inducible gene therapy were acute phase response (APR) promoters (Varley et al. 1995). The promoters of Serum Amyloid A3 (SAA3) and complement factor 3 (C3) were coupled to the luciferase reporter gene in an adenoviral vector and injected in the mouse tail vein. After injection of the bacterial lipopolysaccharide (LPS), luciferase activity increased in liver homogenates transduced with SAA3-luc and C3-luc. The increase was not observed with the constitutive active cytomegalovirus (CMV) promoter. The C3 promoter has been used to express the anti-inflammatory IL-10 in the streptococcal cell wall (SCW) model of arthritis (Miagkov et al. 2002). IL-10 was expressed after inflammatory stimulation and could reduce the influx of immune cells. The same promoter was used by our group to successfully express IL-1Ra to diminish collagen-induced arthritis (CIA) (Bakker et al. 2002). Although these two studies are promising for the gene therapy in RA, both studies used the C3 promoter in combination with the HIV transactivator of transcription (Tat) to amplify the transgene expression and the Tat protein can elicit an immune response and provide unwanted transactivation (Fulcher and Jans 2003).

Our group has also further investigated the SAA3 promoter. Murine joints received intra-articular injections with lentiviral SAA3-luc and the promoter activity was strongly increased after SCW-induced arthritis

(Geurts et al. 2011). *In vitro*, the promoter response was stronger in primary synovial fibroblasts with a high inflammatory phenotype compared to a low inflammatory phenotype. Moreover, a stable SAA3-luc cell line could distinguish between serum from RA patients and healthy control serum.

An additional inducible promoter has highlighted the importance of inducible gene therapy compared to constitutive transgene expression. The human IL-1 enhancer element was coupled to the human IL-6 promoter to provide inducible expression of the chondroprotective IL-4 in CIA (Geurts et al. 2007). Constitutive expression of IL-4 also could protect the cartilage, but the excess of IL-4 also increased inflammation. In a more recent study, this engineered promoter was cloned in front of the IL-10 gene and used to transduce Hematopoietic Stem Cells (HSC's). Systemic injection of the transduced HSC's ameliorated CIA (Henningsson et al. 2012). Several other promoters derived from genes have proven to be suitable for inducible gene therapy in animal models of arthritis. Garaulet and colleagues have shown that a lentivirus with the E-selectin (ESEL) promoter in combination with the IL-10 transgene could provide increased levels of IL-10 after inflammatory stimulation when injected intraplantar in the murine hind paw (Garaulet et al. 2013). Intraplantar treatment with the ESEL-IL10 lentiviral vector could reduce paw swelling in zymosan-induced arthritis (ZIA).

Inducible promoters have also been tested in the context of genetic synovectomy based on principles developed for oncolytic viruses. The telomerase reverse transcriptase gene (TERT) shows increased expression in RA synovial fibroblasts compared to dermal fibroblasts (Chen et al. 2009). The TERT promoter has been used to create a conditionally replicating adenovirus (ad.GS1), which is dependent on TERT promoter activation for replication. The Ad.GS1 could specifically deplete RA synovial fibrobasts in the rat ankle and ameliorate collagen-induced arthritis.

Which inducible promoter performs best in human cells still remains to be determined. Not only the fold induction compared to the healthy situation is important, but the transcriptional activity after activation needs to be sufficient to produce adequate levels of the desired transgene. Which promoter works best is thus also dependent on the therapeutic efficacy of the transgene. In addition, for transgenes with a high risk of side effects, a low basal promoter activity during disease remission is essential.

The most well-studied artificial inflammation-responsive promoter for RA is the minimal CMV-promoter combined with 6 tandem repeats of the nuclear factor kappa-B (NFκB) transcription factor. The first therapeutic benefit was observed using an rAAV5 coding for soluble TNFα receptors (Khoury et al. 2007). Both the

incidence and severity of CIA were reduced after injections in the murine knee and ankle joints. In contrast to a constitutive promoter, the 6xNFκB promoter activity peaked at day 7 after an LPS injection, after which the activity decreased and could be re-activated by a second LPS-injection after 9 weeks. In the same month, a second paper was published describing the application of the rAAV5 vector with the 6xNFκB-promoter. In this study, beneficial effects were found in the rat adjuvant arthritis (AA) model after inducible expression of the immunomodulatory Beta interferon (IFN-β) (Adriaansen et al. 2007, Adriaansen et al. 2007). Interestingly, paw swelling was only reduced in the group treated with inducible IFN-β therapy and not after high constitutive expression of IFN-β, possibly because of lower absolute expression. The rAAV5-NFκB-IFNβ (ART-I02) vector has been further tested and found to be effective in fibroblast-like synoviocytes (FLS) of several species (Aalbers et al. 2015). In addition, the vector was found to be safe in rhesus monkeys with CIA (Bevaart et al. 2015). A non-significant reduction in inflammation was observed after treatment of hand joints, although the vector induced rAAV5-neutralizing antibodies. Currently, a Phase I clinical trial is planned with this vector by the Dutch company Arthrogen, the first gene therapy trial for RA to use an inducible promoter.

Compared to RA, far less effort has been put in finding inducible promoters for OA. The current focus of gene therapy for OA is on finding the best transgene, rather than fine-tuning the expression of successful transgenes with inducible promoters. Several inducible promoters for OA have already been investigated, but these were mainly based on sensitivity to inflammatory triggers, rather than OA-specific processes. Examples of these promoters are the COX-2 promoter (Rachakonda et al. 2008) and the MMP-9 promoter combined with several NF-κB binding sites (Campbell et al. 2005).

Personalized gene therapy

Analysis of gene expression profiles of synovial membranes of RA, OA and healthy controls have yielded (in addition to pathways that were differentially affected between diseases) pathways that showed a large patient-to-patient variation (Huber et al. 2008). For example, genes in the B-cell receptor signaling pathway and the VEGF signaling pathway showed significant differences between RA patients. OA patients had variable expression of genes of genes involved in, for instance, oxidative phosphorylation and MAPK signaling. An earlier study on synovial gene expression already found that a group of RA patients showed increased expression of inflammatory genes, whereas a second group of RA patients had increased expression

of genes associated with tissue remodeling (van der Pouw Kraan et al. 2003).

The large inter-individual differences warrant a patient-specific treatment. Ideally, this can be achieved using inducible promoters and *in vitro* cultures can be the model for this challenge. A previous study from our group focused on the inflammatory phenotype of primary RA and OA synovial fibroblasts in culture. The RA and OA samples seemed to be intermingled when the samples were clustered based on the expression of inflammation-associated genes (Geurts et al. 2011). Interestingly, primary fibroblasts with a high inflammatory phenotype transduced with the Saa3-promoter luciferase reporter showed an increased response to cytokine stimulation compared to primary fibroblasts with a low inflammatory phenotype.

Monolayer cultures of synovial fibroblast are a relatively easy *in vitro* model to test a personalized gene therapy approach. However, in monolayer the cells have no interaction with the surrounding matrix which greatly changes their behavior. One solution to preserve the cell interactions is by using tissue explants derived from arthroscopic biopsies or tissue samples remaining after joint replacement surgery. Disadvantages of this method are a high variability between samples and the fact that many disease processes (eg. hyperplasia, fibrosis) have already occurred irreversibly in this material. The response of targeted gene therapy and the effect of the transgene on synovial pathology can therefore better be tested on an human synovial membrane model. The first artificial synovium was created by mixing a synovial cell suspension obtained by collagenase digestion with a fibrin matrix (Franz et al. 2001). Under correct culture conditions, the synovial fibroblasts remained viable. The first attempt to create an *in vitro* synovial lining was by mixing synovial fibroblasts with Matrigel, a substance that contains extracellular matrix proteins from Englebreth-Holm-Swarm (EHS) tumors in mice, containing many basement membrane proteins (Hughes et al. 2010). The Matrigel is liquid at low temperatures and becomes solid at room temperature. Interestingly, when Matrigel droplets mixed with synovial fibroblasts are cultured, the synovial fibroblasts migrated toward the surface of the Matrigel droplet and form a structure that resembles a synovial lining (Kiener et al. 2010). The cells on the surface expressed Proteoglycan-4 (PRG-4), which is a marker for synovial fibroblasts. In addition, prolonged exposure to TNFα could induce hyperplasia, which strongly mimics the situation of the human synovial lining. Good 3D models of joint structures can be used to study the changes that occur during joint diseases and can provide a higher predictable value for the effects of a therapy in clinical trials. Because advanced models are pivotal to test the potential of gene therapy in complex human tissue before proceeding to a clinical trial, we aim in this thesis to improve the Matrigel-

based 3D culture of human synovium.

Outline of this thesis

Rheumatoid arthritis (RA) and osteoarthritis (OA) are rheumatic diseases for which no cure is available yet. The aim of this thesis is to develop a gene therapy for the treatment of RA and OA. Gene therapy can allow the cells of a transduced joint to produce anti-rheumatic proteins. For RA, several successful protein-based therapies are already available and gene therapy can improve the treatment by targeting joints refractory to medication and potentially reduce side effects, while requiring fewer injections. In addition, the expression of the therapeutic transgene can be regulated by disease-inducible promoters to provide protein production only during flares of active disease. This can further reduce the off-target exposure to the therapy and potentially limit side effects. The experimental approach followed in this thesis is shown in Figure 2.

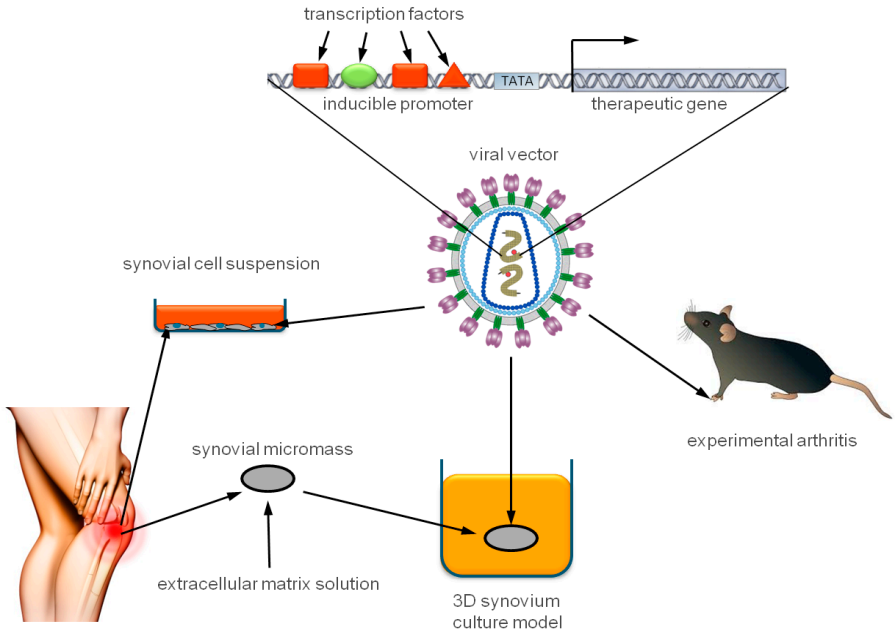

Figure 2: General experimental approach described in this thesis. The focus of this thesis is on applying inducible gene therapy for arthritic diseases. The gene therapy is based on a viral vector that includes an inducible promoter with transcription factor binding sites for both pro-inflammatory (red) and anti-inflammatory (green) transcription factors to fine-tune the expression of a therapeutic transgene. The viral vector is be applied in experimental arthritis models and in *in vitro* cultures of primary cells derived from patient tissue. The therapies are tested in both synovial cell suspensions and the 3-dimensional model of the synovial lining.

In **Chapter 2** we start our quest by testing inducible promoters for the treatment of experimental RA. *Saa3* and *Mmp13* were upregulated during experimental RA and the promoters of these genes showed high activity in the murine joint during the early and the late phase of inflammation respectively. When these promoters were used to drive the expression of the anti-inflammatory Interleukin-10 (IL-10), the inflammation and cartilage damage were significantly reduced, showing the potential of IL-10 and the inducible promoters.

For OA, there is still a quest to find a functional compound that can interfere with the disease process. Based on current insights, the reduction of inflammation and cartilage damage might reduce the progression of OA. A protein with several functions that we speculate might reduce OA pathology is TNFα-stimulated gene 6 (TSG-6, TNFAIP6). In **Chapter 3**, we therefore switch to the treatment of experimental osteoarthritis and test the effects of TSG-6 overexpression. In an *in vitro* validation test, lentiviral TSG-6 overexpression could inhibit bone resorption by osteoclasts. However, when tested *in vivo*, the TSG-6 gene therapy was not able to reduce inflammation or cartilage damage. Instead, increased ectopic bone formation was observed in the medial collateral ligament. This was unexpected and hints at the involvement of TSG-6 in OA disease pathology. Based on these results, we do not recommend TSG-6 as a therapeutic agent for OA gene therapy.

After having established the efficacy of disease-regulated IL-10 therapy in experimental RA, we desired to explore the gene therapy effects in human *in vitro* systems. We first set out to tackle two important challenges faced when applying *in vitro* models based on primary human (patient) material. Firstly, primary material is available at irregular intervals. Patients are not operated simultaneously and experiments that require multiple donors cannot be concurrently executed. In **Chapter 4**, we seek to improve the possibilities for *in vitro* studies with primary OA synovial tissue by validating a protocol for cryopreservation. By applying the correct protocol based on CS10 cryopreservation medium, the viability of a synovial tissue biopsy after cryopreservation can be maintained. In addition, there is no significant induction of stress-related gene expression and the expression of OA-related gene expression, which represents the OA phenotype, is maintained. Moreover, the ability to produce cytokines after pro-inflammatory stimulation is not significantly affected. This paves the way to establish a biobank of OA in which synovial tissue can be stored. To even further improve the *in vitro* possibilities to study gene therapy in the complex tissue of the synovium *in vitro*, we add to the development of the 3D synovial micromass model, based on human primary synovial cells that can form a lining similar to the synovial membrane. **Chapter 5** focuses on the characterization of the synovial

micromass model. In this model, a synovial cell suspension is mixed with Matrigel fluid, which becomes solid at 37°C and contains different basement membrane and extracellular matrix proteins. We observe the migration of synovial fibroblast-like cells and macrophage-like cells to the surface to form a lining layer reminiscent of the synovial membrane. Several other cell types also appear in the micromass, but do not migrate. After stimulation with TNFα for 3 weeks, synovial hyperplasia occurs, which is similar to the situation *in vivo*. Interestingly, the synovial micromasses can be cryopreserved without a loss of viability or inflammatory response.

After studying inducible gene therapy in experimental RA and improving the *in vitro* research options for experiments based on primary human material, the inducible expression of anti-inflammatory IL-10 is extended to the human RA situation in **Chapter 6**. Based on microarray data of RA and control synovium, the promoter of the *CXCL10* gene was selected for IL-10 expression. The *CXCL10* promoter was responsive to inflammatory stimuli and serum from RA patients. When a cell suspension of RA synovial cells was transduced with the CXCL10-IL10P lentiviral vector, the production of IL-10 was increased after stimulation with pro-inflammatory factors. The increased IL-10 levels could reduce the production of pro-inflammatory cytokines by the cells. We ultimately apply the CXCL10p-IL10 vector in the micromass model in **Chapter 7**. OA micromasses are transduced with the lentiviral CXCL10P-luciferase vector. Using confocal imaging, we show that the lining can be transduced, which is similar to findings in viral vector-transduced murine joints. The *CXCL10* promoter was responsive to pro-inflammatory triggers and could provide a disease-responsive production of IL-10. When treated with the CXCL10P-IL10 vector, the production of pro-inflammatory factors was reduced. This shows that the CXCL10P-IL10 vector can suppress the inflammation in an OA synovial lining model, indicating that CXCL10P-IL10 might be an interesting candidate to modify the disease course in OA.

A discussion and future perspectives for the micromass model, cryopreservation, gene therapy and inducible promoters for RA and OA are provided in **Chapter 8**.

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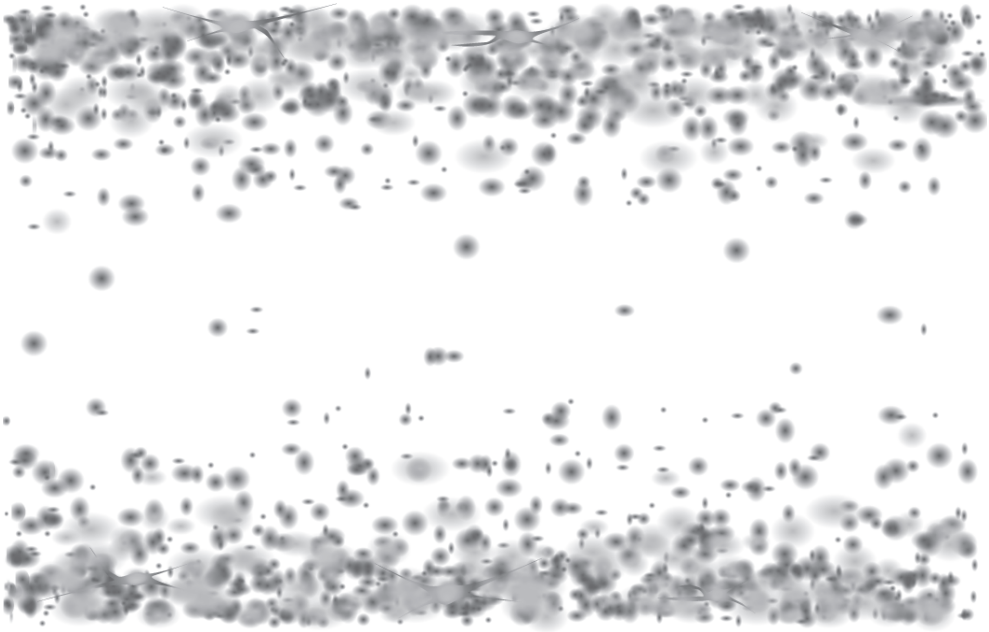

Chapter 2

Disease-regulated local IL-10 gene therapy diminishes synovitis and cartilage proteoglycan depletion in experimental arthritis

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Objectives:
Rheumatoid arthritis (RA) is a chronic destructive autoimmune disease, but the course is unpredictable in individual patients. An attractive treatment would provide a disease-regulated therapy that offers personalized drug delivery. Therefore, we expressed the anti-inflammatory interleukin-10 (IL-10) gene under the control of inflammation-dependent promoters in a mouse model of arthritis.

Methods:
Proximal promoters of *S100a8, Cxcl1, Mmp13, Saa3, IL-1b*, and *Tsg6* were selected by whole genome expression analysis of inflamed synovial tissues from arthritic mice. Mice were injected intra-articularly in knee joints with lentiviral vectors expressing a luciferase reporter or the therapeutic protein IL-10 under control of the Saa3 or Mmp13 promoter. After 4 days, arthritis was induced by intra-articular injection of streptococcal cell walls (SCW). At different time points after arthritis induction, in vivo bioluminescent imaging was performed and knee joints were dissected for histological and RNA analysis.

Results:
The disease-regulated promoter-luciferase reporter constructs showed different activation profiles during the course of the disease. The *Saa3* and *Mmp13* promoters were significantly induced at day 1 or day 4 after arthritis induction respectively and selected for further research. Overexpression of IL-10 using these two disease-inducible promoters resulted in less synovitis and markedly diminished cartilage proteoglycan depletion and in upregulation of IL-1Ra and SOCS3 gene expression.

Conclusions:
Our study shows that promoters of genes that are expressed locally during arthritis can be candidates for disease-regulated overexpression of biologics into arthritic joints, as shown for IL-10 in SCW arthritis. The disease-inducible approach might be promising for future tailor made local gene therapy in arthritis.

Introduction

Arthritis is a debilitating disease which affects about 1% of the world population. The course of the disease can be intermittent, with episodes of inflammation and remission. Conventional treatments nowadays consist of repeated systemic administration of biological drugs, such as tumor necrosis factor alpha (TNF-α) blockers, which continuously suppress the immune system. This kind of treatment gives rise to different side effects, like infections and the formation of anti-drug antibodies.[1] Local gene therapy for arthritis, with the use of disease-regulated promoters, represents a promising alternative for coping with side effects of the conventional treatments. Using gene therapy via viral expression systems, the patient can be treated for a prolonged time, by only a single intra-articular injection. Nathwani and co-workers[2] already showed this by treating hemophilia B patients using viral overexpression of factor IX.

Previously, we constructed different disease-regulated promoters from genes differentially regulated during collagen-induced arthritis in mice, selected via a computational approach[3]. These disease-regulated promoters respond to transcription factors activated during inflammation, restricting the production of the transgene to episodes of inflammation. Several promoters showed an inducible profile in lentivirally transduced macrophage and fibroblast cell lines after in vitro stimulation with LPS. In addition, the Saa3 promoter was able to detect differences between high and low inflammatory profiles in synovial fibroblasts from rheumatoid arthritis (RA) and osteoarthritis patients.[4] This proved the inducibility and sensitivity of this promoter to disease activity.

Interleukin-10 (IL-10) is a good candidate transgene to suppress arthritis using disease-regulated promoters. IL-10 is a broad spectrum anti-inflammatory cytokine and is produced by different immune cells, like Th1 and Th2 cells, B cells, monocytes and macrophages. Inhibition of several pro-inflammatory cytokines has been reported; effects were seen on interleukin-1 (IL-1) and TNF-α.[5,6] IL-10 is also known to induce the production of interleukin-1 receptor antagonist (IL-1Ra) and suppressor of cytokine signaling-3 (SOCS3).[7,8] Nonetheless, several clinical studies showed lack of efficacy using IL-10 supplementation and even increased disease activity as compared to placebo treated RA patients has been reported[9]. Because the half-life of IL-10 is short, around 1.1 to 2.6 hours,[8] patients had to be injected either intravenously or subcutaneously 3 times a week. Furthermore, in different cohorts, IL-10 treated patients were at risk of developing anemia. This paved the way for developing a more effective F8-IL-10 fusion protein, which targets the systemically

delivered IL-10 to the fibronectin deposits in the arthritic joints.[10]

Unnecessary off-target effects can also be avoided by using disease-regulated gene therapy to limit the height and time of exposure to IL-10 as we previously have shown for IL-4, a cartilage protective cytokine that has proinflammatory properties *in vivo.*[11] We previously showed feasibility of the disease-regulated IL-10 therapy by transplantation of lentiviral transduced haematopoeitic stem cells.[12] This led to production of IL-10 in B and non-B APC cells in lymph nodes and clear-cut reduction of collagen-induced arthritis (CIA). An effect also shown by Tanaka *et al.*[13] where they found effects of IL-10 protein treatment on CIA. A reduction in the incidence and score of the arthritis was found, together with a significant reduction of IL-6 by macrophages. The effect of local IL-10 treatment using this disease-regulated gene therapy concept has not been explored and could be very promising.

The streptococcal cell wall (SCW) model was chosen, because repeated intraperitoneal IL-10 injections showed a significant reduction in joint pathology.[14] Also, different processes of influx of inflammatory cells in the joint cavity, synovitis and proteoglycan depletion occur sequentially at different time points, resulting in a predictable disease course. Because we expected the disease-regulated promoters to respond differently to the these events, this model was chosen to elucidate the different kinetics of the disease-regulated promoters.

Hence, in this study, we set out to select the suitable disease-regulated promoter according to the profiles of the different promoters during the SCW-induced arthritis model. We showed that local disease-dependent overexpression of IL-10 diminished proteoglycan depletion and synovitis and is therefore proven to be a suitable strategy to suppress disease activity.

Material and Methods

Animals

Male 12 week old C57Bl/6N mice were obtained from Janvier (Le Genest Saint Isle, France). During virus experiments, mice were housed in HEPA filtered individually ventilated cages and were fed a standard diet with food and water *ad libitum*. All *in vivo* studies complied with national legislation and were approved by the local authorities of the Care and Use of Animals.

Plasmids

Recombinant lentiviral vectors were generated using the third-generation self-inactivating transfer vector pRLL-cPPT-PGK-mcs-PRE-SIN (PGK-empty) containing the human phosphoglycerate kinase (PGK) promoter (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). Construction of promoter-luciferase constructs was done as described elsewhere.[3] For construction of pRLL-cPPT-PGK-IL10-PRE-SIN, pRLL-cPPT-Saa3-IL10-PRE-SIN and pRLL-cPPT-MMP13 -IL10-PRE-SIN, mIL-10 was amplified from pIG1-IL1e-IL6p-IL10, a construct used by Henningsson and collegues,[12] using primers FW 5'- tttgctagctccaccatgcctggctc-3' and RV 5'-aaacatatgttagcttttcattttg-3' introducing an NheI and NdeI site, and ligated into the promoter constructs.

Lentiviral vector production

Lentiviral vector production was performed as described previously[3] with co-transfection of packaging and expression plasmids at half concentration. For the *in vivo* studies, knee joints were injected with 300 ng $p24^{gag}$ equivalents lentivirus in a total volume of 6 µl.

Cell culture

Mouse embryonic fibroblasts (NIH-3T3) and macrophages (RAW 264.7) were cultivated in DMEM with 1 mM pyruvate, 40 µg/ml gentamycin, and 5% or 10% fetal calf serum (FCS) respectively. Cells were kept at 37˚C in a humid atmosphere containing 5% CO_2.

In vitro luciferase measurements

Cells were seeded at 3×10^4 cells per well in a white clear bottom Costar 96 wells plate (Corning, NY). After one day, cells were transduced with 50 ng $p24^{gag}$ equivalents lentivirus in 50 µl medium supplemented with 8 µg/ml polybrene (Sigma) for 4 hours at 37˚C. Two days after transduction, cells were serum starved (1% FCS) for one day and subsequently stimulated with 10 ng/ml recombinant mIL-10 (R&D systems) or a combination of 10 ng/ml Pam3Cys (EMC Microcollection) and 100 ng/ml *E.Coli* LPS (Sigma) for indicated hours. Thereafter, cells were lysed in water and luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar, BMG, Offenburg, Germany).

Preparation of streptococcal cell walls and induction of arthritis

Strepococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously.[15] Uni- or bilateral arthritis was induced four days after transduction with the lentivirus by injection of 25µg SCW in 6µl of phosphate buffered saline (PBS) into the joint cavity of the knee.

In vivo bioluminescence imaging

At indicated time points after SCW injection, *in vivo* bioluminescent imaging was performed (n=6 mice per inducible promoter) on an IVIS Lumina system (Caliper Life Sciences, Hopkinton, MA, USA), 10 minutes after intraperitoneal injection of 150 mg/kg D-Luciferine (Caliper Life Sciences) dissolved in PBS. Mice were anesthetized with 2,5% isoflurane/oxygen, shaved, placed on their back in the light tight chamber and imaged for 4 minutes with a sensitive CCD camera. Images were analyzed using the Living Image 3.0 software (Caliper Life Sciences). Two-dimensional regions of interest (ROIs) were drawn around the knee joints. Luciferase activity is presented in photons emitted per second per square cm.

Histology

In total, 9 knee joints per inducible promoter per time point were used. Histological sections of knee joints were analyzed for synovitis, and proteoglycan depletion as described earlier.[16]

RNA isolation and quantitative PCR analysis

One synovial tissue sample from the lateral side and one from the medial side of the knee joint were isolated using a 3 mm biopsy punch (Stiefel, Wachtersbach, Germany) and pooled. In total, 9 knee joints per promoter per time point were analyzed. Total RNA was extracted from the tissue homogenates and from cells using TRI reagent (Sigma) according to manufacturer's protocol. Isolated RNA was treated with DNAse followed by reverse transcription of 1µg RNA into cDNA using Moloney murine leukemia virus reverse transcriptase 0.5µg/µl oligo(dT) primers, and 12.5mM dNTPs (Invitrogen). Quantitative real-time PCR was performed using the StepOnePlus sequence detection system (Applied biosystems). PCR was performed in a total reaction volume of 12.5 µl consisting of appropriate cDNA, 5 µM forward and reverse primer and SYBR green PCR master mix (Applied biosystems). The PCR protocol consisted of 2 min at 50°C and 10 min of 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The cycle threshold value (Ct) of genes of interest were compared to the Ct of reference gene glyceraldehyde-3-phophate

dehydrogenase (GAPDH) (delta Ct). Primer sequences are listed in Table S1.

Blood serum cytokine measurement

KC (the murine homologue of IL-8) levels in the blood serum, were determined on a Luminex-100 system (Luminex Corp) using a bead-based multiplex immunoassay (Milliplex, Merck Millipore). Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistics

Statistical analysis was performed using students' t-test, 1way ANOVA, Pearson correlation or Mann-Whitney U test (GraphPad Prism, V 4.0). A p-value of <0.05 was regarded as significant.

Results

Selection of a promoter for local gene therapy.

Luciferase reporter expression profiles of 6 different disease-regulated promoter reporters were made during SCW arthritis, using bioluminescence imaging **(Fig. 1A,B)**. All 6 promoters showed different activation kinetics during the acute model of arthritis. The *Saa3* promoter showed the highest fold increase (127-fold) with an early activation at day 1 after arthritis induction. The *Mmp13* promoter response showed a delay and was activated at day 4 and stayed high up to 7 days. All other promoters peaked at day 4 or 7 after SCW injection. Because of these profiles, the *Saa3* and *Mmp13* promoters seemed interesting candidates for local gene therapy using the anti-inflammatory IL-10 transgene. To ascertain that both promoters responded only to the local arthritic process we first tested the *Saa3* and *Mmp13* promoter responses to IL-10, our transgene of choice. Mice were co-injected with PGK-IL10 and Saa3-luciferase or Mmp13-luciferase and the luciferase signal was measured at the same time points of SCW-arthritis **(Fig. 2)**. The *Saa3* and *Mmp13* promoters were still activated at day 1 and 4 respectively, indicating that the inducible promoters are not responsive to IL-10 *in vivo*. Therefore, no positive feedback loop will be created by the promoters that might uncouple their regulation by the local inflammatory process. This finding was supported *in vitro,* where likewise no effects of IL-10 were found on the activation of the *Saa3* promoter by SCW in fibroblasts and macrophages, both important cell types in the synovial lining **(Fig. S1)**. Our *Mmp13* promoter construct is not responsive in both cell-types to the stimuli tested

as we have shown before,[3] therefore an IL-10 effect could not be demonstrated (data not shown).

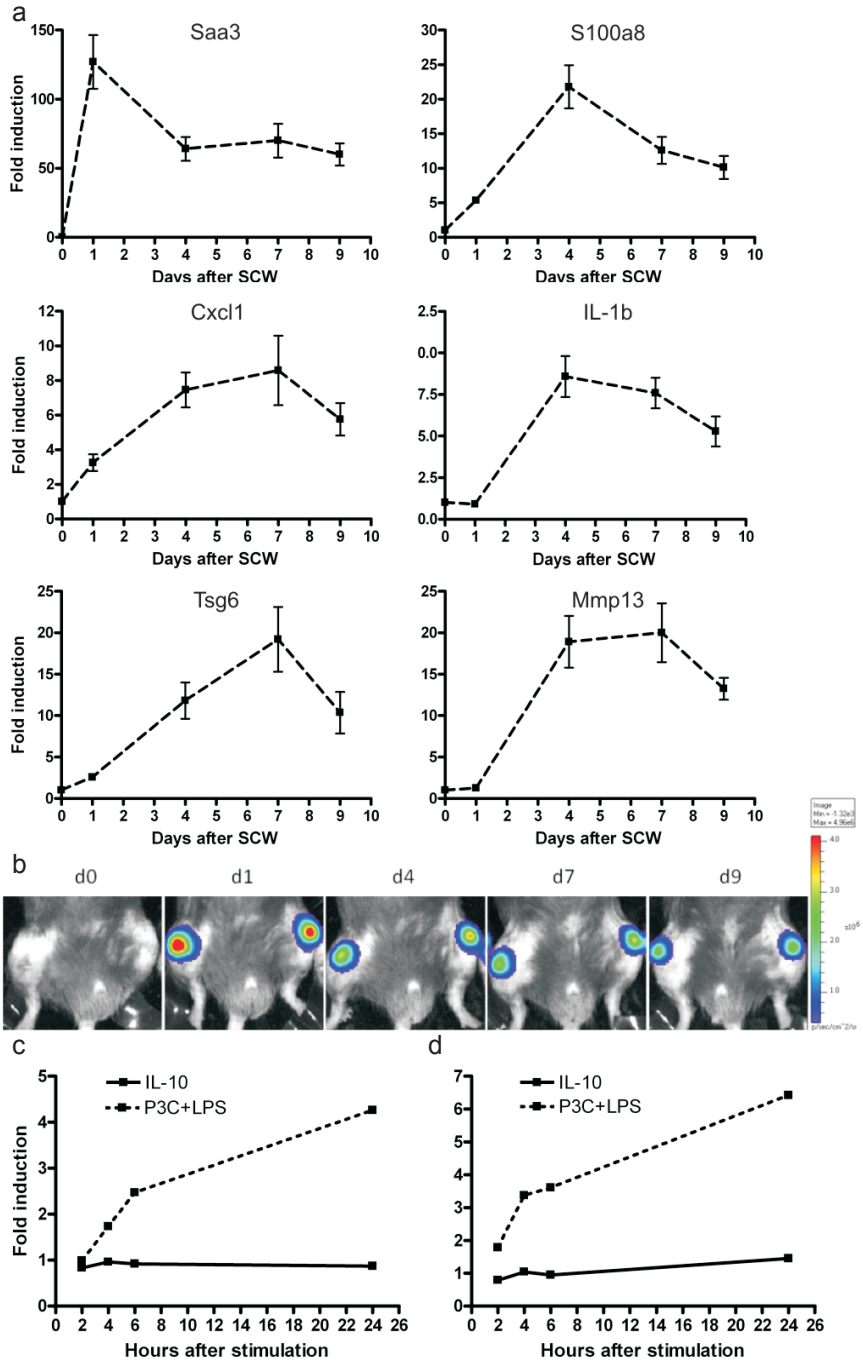

Figure 1. Profiling of the different disease-regulated promoters using bioluminescent imaging.
A) Both knee joints of 6 C57Bl/6N mice per inducible promoter were transduced with 300 ng $p24^{gag}$ lentiviral vector containing either the *Saa3, S100a8, Cxcl1, IL-1b, Tsg6* or *Mmp13* proximal promoter with luciferase reporter gene. Four days after transduction, the knee joints were injected with 25 µg SCW to induce arthritis. At day 0, 1, 4, 7 and 9, mice were imaged in a light tight chamber 10 minutes after luciferin injection. Luciferase activity was quantified and plotted as fold induction compared to day 0. **B)** Representative images of the bioluminescent imaging of the *Saa3* promoter in one mouse at day 0, 1, 4, 7 and 9. Depicted as mean±SEM; n=12.

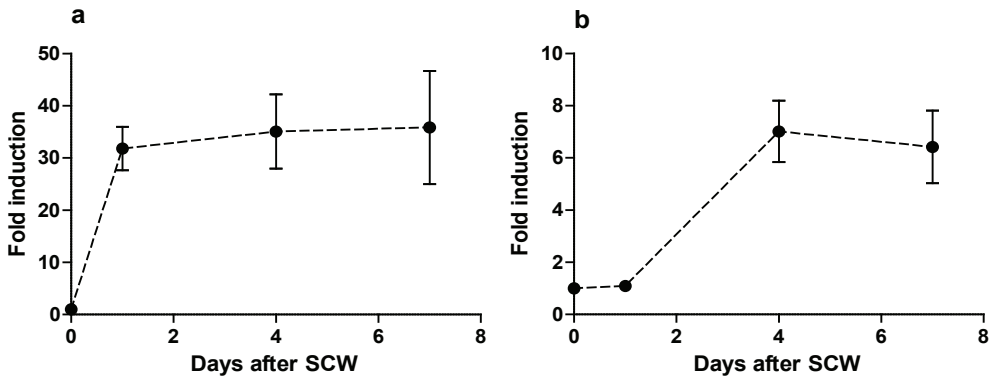

Figure 2. Profiling of the disease-regulated Saa3 and Mmp13 promoter in SCW arthritis in the presence of local IL-10 expression.
Both knee joints of 6 C57Bl/6N mice per inducible promoter were transduced with 300 ng p24gag lentiviral vector containing PGK-IL-10, cotransfected with either 300 ng p24gag lentiviral vector Saa3-luciferase **(A)** or Mmp13-luciferase **(B)**. Four days after transduction, the knee joints were injected with 25 4g SCW to induce arthritis. At day 0, 1, 4, 7 and 9, mice were imaged in a light tight chamber 10 minutes after luciferin injection. Luciferase activity was quantified and plotted as fold induction compared to day 0. Depicted as mean±SEM; n=6.

IL-10 is upregulated by the *Saa3* and *Mmp13* promoters during arthritis.

Next, the disease-regulated expression of the IL-10 transgene under the control of the inducible promoters was examined **(Fig. 1A)**. Mice were i.a. injected in both knee joints with the lentiviral construct expressing IL-10 under the control of the constitutive PGK or the inducible *Saa3* or *Mmp13* promoter. One knee joint was injected with 25 µg SCW and the contralateral knee joint with PBS. Subsequently, IL-10 mRNA expression was measured in both knee joints at day 1 after the intra-articular injections. The constitutive PGK promoter showed

the same extent of IL-10 expression in both the PBS injected control joint as well as the SCW injected arthritic knee joint, while the inducible *Saa3* promoter showed a significant inducible expression in the arthritic knee joint compared to the contralateral knee joint **(Fig. 3A,B)**. The *Mmp13* promoter showed a slightly higher activity in the contralateral joint compared to *Saa3* **(Fig. 3B,C)**. At day 4 and 7, IL-10 expression was upregulated by all three promoters, compared to the empty control treated animals **(Fig. 3D,E)**. This confirms that the *Saa3* and *Mmp13* promoters are activated by inflammation after induction of arthritis.

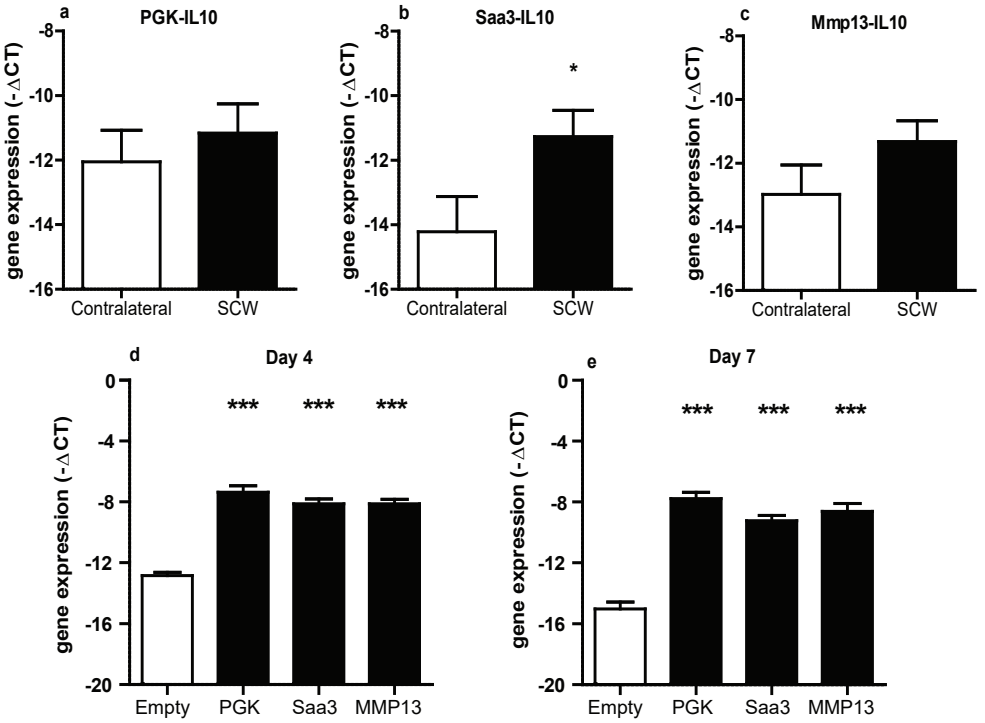

Figure 3. IL-10 gene expression analysis of synovial tissue from knee joints after arthritis induction in joints with IL-10 overexpression.

Both knee joints received intra-articular injections with lentiviral PGK-IL10, Saa3-IL10 or Mmp13-IL10. Arthritis was induced in one knee joint 4 days after transduction. After 1 day, gene expression analysis was performed for IL-10 in synovial tissue from both the arthritic and non-arthritic contralateral knee joint **(A-C)**. IL-10 expression was also measured in synovial tissue from PGK-empy, PGK-IL10, Saa3-IL10 and Mmp13-IL10 injected arthritic knee joints at day 4 **(D)** and day 7 **(E)**. Gene expression is shown as -delta Ct, corrected for GAPDH. Depicted as mean±SEM; n=9. * = $p<0.05$, *** = $p<0.0001$ versus control Group empty when using a paired t-test (a-c) or a 1way ANOVA (D,E).

Disease-dependent IL-10 expression decreases synovitis and proteoglycan depletion.

To test the therapeutic effects of disease-regulated IL-10 expression, mice were treated locally with IL-10 under control of the *Saa3* or *Mmp13* promoter. After 4 days, SCW arthritis was induced. Mice were sacrificed for histological- and gene expression analysis at 1, 4 or 7 days after arthritis induction. Synovitis was significantly reduced in IL-10 treated joints at day 4 after arthritis induction **(Table 1, Fig. 4A-D)**.

Proteoglycan depletion was significantly lower in IL-10 treated animals at day 4 and 7 after arthritis induction **(Fig. 4E-H)**. Because the extent of the therapeutic effect on SCW arthritis of the *Saa3* and *Mmp13* promoters were similar, the subsequent *in vivo* experiments were performed using the *Saa3* promoter.

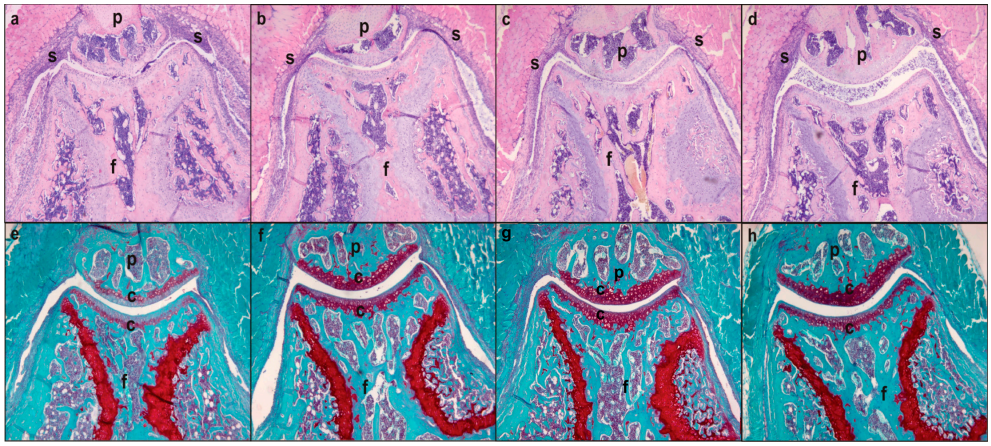

Figure 4. Histology of day 4 and 7 after arthritis induction in joints with IL-10 overexpression.

Histology of knee joints using haematoxylin-eosin staining for analysis of inflammation and safranin-O staining for analysis of cartilage proteoglycan depletion. Knee joints were injected with PGK-empty **(A,E)**, PGK-IL10 **(B,F)**, Saa3-IL10 **(C,G)** or Mmp13-IL10 **(D,H)**. Histology from day 4 **(A-D)** or day 7 **(E-H)** after arthritis induction is shown. Representative pictures from mice described in table 1 are depicted. p=patella, f=femur, s=synovium, c=cartilage.

Table 1. Histological scoring after local inducible IL-10 gene therapy in SCW-induced joint pathology.

Histological scoring of exudates (exu), synovitis (syn) and proteoglycan depletion (PG depl) at day 1, 4 and 7 after arthritis induction showed a significant decrease in synovitis and proteoglycan depletion after IL-10 overexpression. Depicted as median±interquartile range. n=6 mice. * = p<0.05, ** = p<0.01, *** = p<0.0001 statistically significant as compared to control group PGK-Empty using a 1way ANOVA.

	Day 1			Day 4			Day 7		
	Exu	Syn	PG depl	Exu	Syn	PG depl	Exu	Syn	PG depl
PGK Empty	3.0 (2.6-3.0)	1.0 (0.6-1.0)	1.0 (0.1-1.5)	1.5 (1.0-1.5)	2.0 (2.0-2.5)	3.0 (2.0-3.0)	0.5 (0.5-0.5)	1.0 (1.0-1.8)	2.0 (1.5-3.0)
PGK IL10	2.3 (2.0-2.9)	1.0 (1.0-1.4)	0.8 (0.1-1.0)	1.5 (1.0-1.8)	1.5** (1.3-2.0)	2.0* (1.5-2.0)	0.5 (0.5-0.5)	1.0 (1.0-1.5)	0.5** (0.0-1.3)
Saa3 IL10	3.0 (2.3-3.0)	1.0 (0.6-1.0)	0.8 (0.5-1.4)	1.0 (0.5-1.5)	1.5** (1.0-2.0)	1.5*** (1.0-1.8)	0.5 (0.0-0.5)	1.0 (0.8-1.3)	0.0** (0.0-1.8)
Mmp13 IL10	2.8 (2.5-3.0)	1.0 (0.6-1.4)	1.0 (0.6-1.4)	2.0 (0.8-2.0)	1.5** (1.0-2.0)	1.5** (0.5-2.3)	0.5 (0.3-0.5)	1.5 (1.0-1.8)	1.0* (0.3-2.0)

Disease-dependent IL-10 upregulation has an effect on protein and gene expression.

Keratinocyte chemoattractant (KC) protein concentration was measured one day after arthritis induction in the serum of Saa3-IL-10 mice. KC is the murine homologue of IL-8, a cytokine involved in recruiting inflammatory cells to invade the joint. A significant reduction in KC concentration from 359±84 pg/ml to 161±32 pg/ml using the *PGK* promoter and to 139±26 pg/ml using the *Saa3* promoter was seen compared to the control group **(Fig. 5)**. In addition, IL-1Ra was upregulated in the synovium at the gene expression level at day 7 in the *Saa3* group, compared to the empty control virus. This effect was also seen on *Socs3* gene expression at day 4 and 7 **(Fig. 6A,B)**. At these time points, IL-1Ra and Socs3 expression was significantly correlated with IL-10 expression **(Fig. 6C)**, indicating that the upregulation is the result of IL-10 expression.

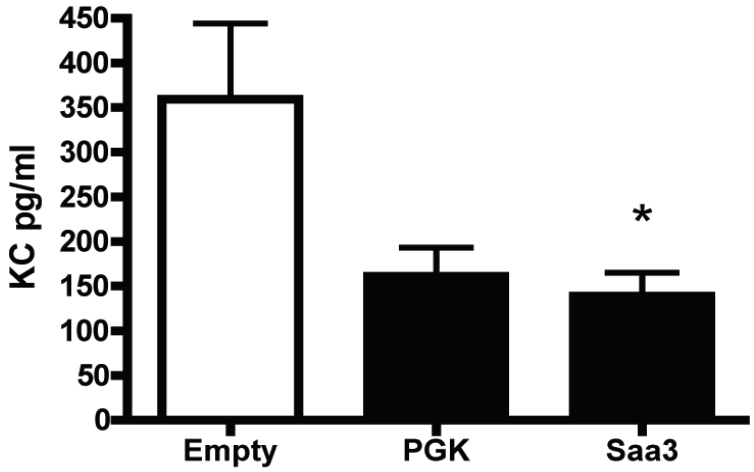

Figure 5. Expression of KC at day 1 after arthritis induction in knee joints with IL-10 overexpression.

KC protein concentration (pg/ml) was measured in blood serum at day 1 after arthritis induction using a bead array. A significant ($p<0.05$) lower concentration was found in the blood serum of mice treated with Saa3-IL10. Depicted as mean±SEM; n=9. * = $p<0.05$ versus control group empty using a 1way ANOVA.

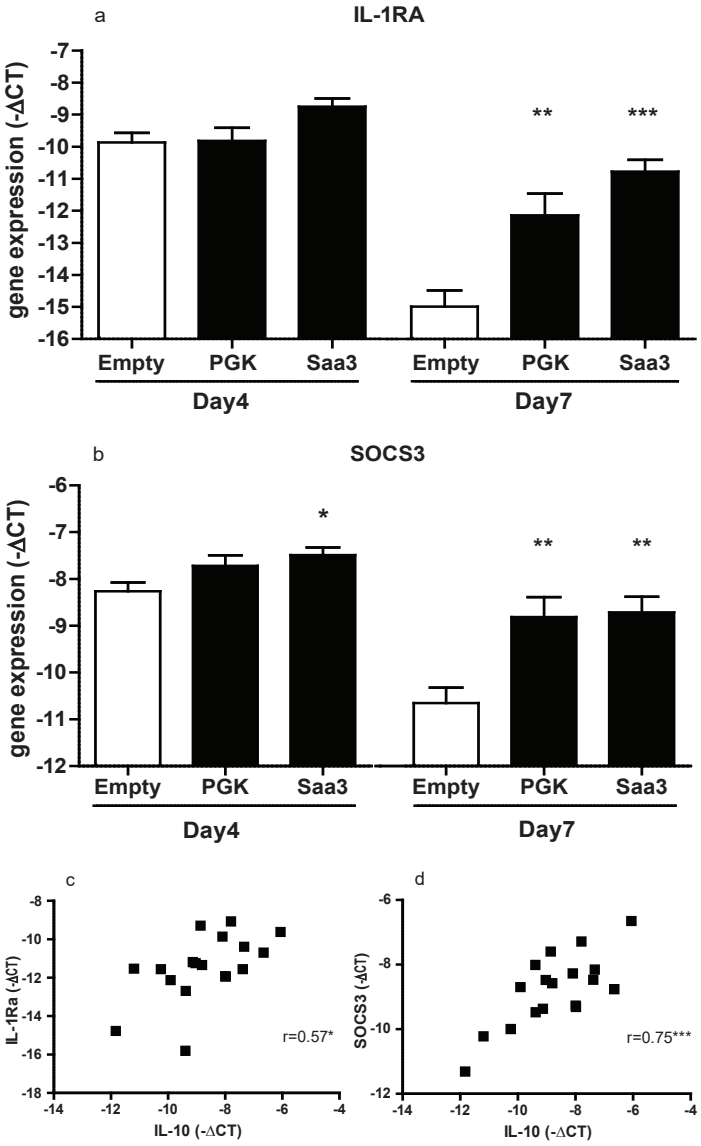

Figure 6. IL1Ra and Socs3 mRNA expression after IL-10 overexpression.

IL1Ra **(A)** and Socs3 **(B)** gene expression in synovial tissue from knee joints was measured by qPCR. Significant upregulation was seen in the PGK-IL10 and Saa3-IL10 treated groups, compared to the PGKempty group. Gene expression is shown as -delta Ct, corrected for GAPDH. Depicted as mean±SEM; n=9. * = p<0.05, ** = p<0.01, *** = p<0.0001 versus control group empty when using a 1way ANOVA. The expression levels at day 7 were correlated with IL-10 overexpression for IL1Ra **(C)** and SOCS3 **(D)**. Depicted as individual joints. * = p<0.05, *** = p<0.0005 using Pearson r correlation analysis.

Discussion

In this study, we have shown that IL-10 under the control of the disease regulated promoters *Saa3* and *Mmp13* can diminish synovitis and cartilage proteoglycan depletion in experimental arthritis. Effects were seen on histology, protein- and gene expression levels.

SCW-induced arthritis is an acute and transient model, which triggers the alternative complement activation cascade to attract neutrophils to the joint, already at day 1 after induction. A peak in synovitis is seen at day 4 after induction and proteoglycan depletion is most severe at day 7. During these stages of the disease, different transcription factors are activated, triggering the recombinant promoters at different time points, resulting in distinct profiles between the different promoters. The *Saa3* promoter contains multiple predicted binding sites for C/EBPβ,[3] which is associated with the acute phase of inflammation.[11] Microarray analysis also revealed a high and quick upregulation of the *Saa3* gene in the knee joints of mice with RA.[3] According to the in vivo profile of promoter activation, *Saa3* is highly upregulated with a 127 fold increase one day after induction of arthritis, as expected for an acute phase protein. In contrast, the *Mmp13* promoter contains at least three functional binding sites for ETV4, which is associated with tissue remodeling and repair mechanisms taking place in the remission phase of arthritis.[17] This explains the observed delay in MMP13-luciferase expression in the short-lasting SCW-arthritis model.

The inducibility of the *Saa3* and *Mmp13* promoters was shown by upregulation of IL-10 in the arthritic knee, compared to the non-arthritic knee. The constitutive *PGK* promoter, on the other hand, did not show inducibility, but a continuous upregulation of IL-10 in both the arthritic and non-arthritic knee. No direct effects of IL-10 on Saa3 promoter activation were found, indicating that the Saa3-IL-10 construct produces no positive feedback loop. Among others, the *Mmp13* promoter was activated at from day 4. The *Tsg6* promoter even showed strongest induction at day 7, indicating that the promoters are activated during different phases in disease. Therefore, promoters can be selected for temporal delivery of therapeutic transgenes to target a specific process during the course of disease, e.g. osteophyte formation or synovial fibrosis. Such stratified approacheswarrant further investigation.

Upregulation of IL-10 was seen at all days after disease induction. The therapeutic effect of IL-10 overexpression was seen on histology: both proteoglycan depletion and synovitis were significantly decreased. This is in line with the results of Lubberts *et al*.[14] where likewise an effect of IL-10 on inflammatory cell influx and proteoglycan depletion was found after repeated

injections of IL-10 during SCW arthritis in mice. In addition, both IL1Ra and SOCS3 were upregulated. These genes are known to be induced by IL-10.[6,7] IL1Ra is known to counteract the detrimental IL-1-dependent production and activation of different MMPs and aggrecanases.[18] By blocking IL-1 with IL-10 induced IL-1Ra, these effects might be inhibited. SOCS3 can inhibit the JAK/STAT pathway and therefore diminish synovitis by, for instance, downregulating IL-6.[7,19]

The neutrophil attracting chemokine KC protein levels were also significantly lower at day 1 after disease induction, which can influence synovitis as well.[20] Arthritis activates the complement cascade, with subsequent release of C5a[21] C5a can activate the p38 MAP kinase pathway[22] and as a consequence KC is upregulated.[23] The p38 pathway can be inhibited by IL-10[24] and therefore influence the expression of KC. Alternatively, IL-10 can induce instability of KC mRNA by repressing the mRNA stabilizing protein HuR.[24,25]

We showed that treatment of an acute joint inflammation with local IL-10 overexpression under the control of disease-regulated promoters, is a promising concept to modulate arthritis progression. By precise timing and prolonged, restricted production at the site of inflammation we might limit adverse IL-10 side effects in RA patients, such as the risk of developing anemia. It has been shown that IL-10 can enhance the FcγRI and IIa expression on monocytes/macrophages resulting in enhanced TNF-α production in response to immune complexes.[9] Although we did not use an antibody-mediated arthritis model, IL-10 in our study did not enhance the expression of both receptors (data not shown). Disease-inducible promoters might be applied for expression of a transgene as a quick response to inflammation, or for supplementing the endogenous production of anti-inflammatory mediators at later time points, as we showed for the *Saa3* and *Mmp13* promoters respectively. This study showed that our previously described computational approach for selecting disease-response promoters combined with thorough *in vivo* validation using bioluminescent imaging of transgene expression results in promising candidates for tailor made/personalized therapy using local delivery of biological genes in arthritis.

Financial support

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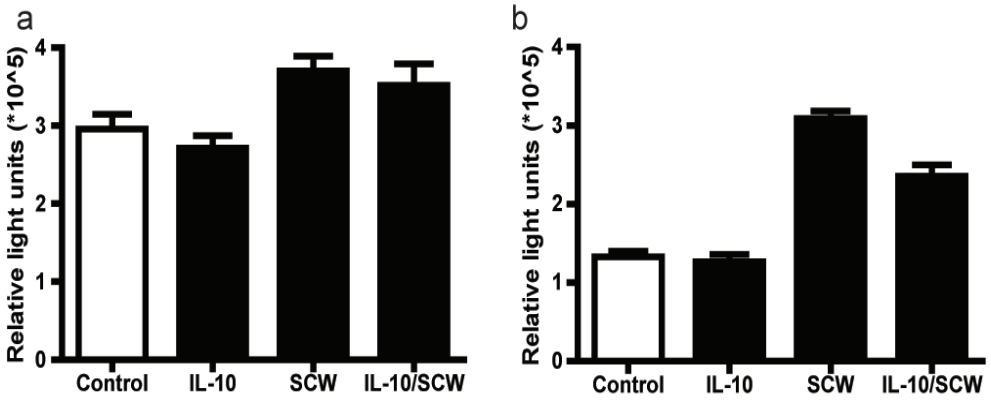
Supplementary information

Supplementary Table S1. Sequences of primers used for quantitative PCR measurements.

Primer	FW	RV
IL-10	ATTTGAATTCCCTGGGTGAGAA	ACACCTTGGTCTTGGAGCTTATTAA
IL-1Ra	CAAGATGCAAGCCTTCAGAATCT	CACCATGTCTATCTTTTCTTCTAGTTTGA
SOCS3	TAGACTTCACGGCTGCCAAC	CGGGGAGCTAGTCCCGAA



Supplementary Figure S1: Luciferase signal in stimulated fibroblasts and macrophages in vitro after transduction with Saa3-luciferase.

NIH-3T3 fibroblasts **(A)** or RAW264.7 macrophages **(B)** were transduced with 50 ng p24gag lentiviral vector containing the Saa3 proximal promoter and luciferase reporter gene. Subsequently, cells were stimulated with either IL-10, SCW or a combination IL-10 and SCW. 6 hours after stimulation, luciferase activity was quantified and plotted as relative light units.

Chapter 3

TSG-6 gene therapy in experimental osteoarthritis results in ectopic bone formation

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Submitted for publication

Objective:
Tumor necrosis factor-inducible gene 6 (TSG-6) has anti-inflammatory and chondroprotective effects in mouse models of inflammatory arthritis. Because cartilage damage and inflammation are also observed in OA, we determined the effect of viral overexpression of TSG-6 in experimental osteoarthritis.

Methods:
Bone marrow-derived cells (BMDCs) were differentiated to multinucleated osteoclasts in the presence of recombinant TSG-6 or after transduction with a lentiviral TSG-6 expression vector. Multi-nucleated osteoclasts were analyzed after tartrate resistant acid phosphatase (TRAP) staining and resorption activity was determined on dentin slices. Collagenase-induced osteoarthritis (CIOA) was induced in C57BL/6 mice after intra-articular injection of an adenoviral TSG-6 or control luciferase expression vector. Inflammation-related protease activity was measured using bioluminescent Prosense probes. After a second adenovirus injection, cartilage damage was assessed in histological sections stained with Safranin-O. Ectopic bone formation was scored in X-ray images of the affected knees.

Results:
TSG-6 did not inhibit the formation of multi-nucleated osteoclasts, but caused a significant reduction in the resorption activity on dentin slices. Adenoviral TSG-6 gene therapy in CIOA could not reduce the cartilage damage compared to the luciferase control virus and no significant difference in inflammation-related protease activity was noted between the TSG-6 and control treated group. Instead, X-ray analysis and histological analysis revealed the presence of ectopic bone formation in the TSG-6 treated group.

Conclusion:
Gene therapy based on the expression of TSG-6 could not provide cartilage protection in experimental osteoarthritis, but instead resulted in increased ectopic bone formation.

Introduction

No disease-modifying treatments are currently available for the treatment of OA and affected end-stage joints are often surgically replaced. OA was once viewed as mechanical wear-and-tear of the cartilage, but in recent years there is increasing evidence that OA is a complex disease affecting the whole joint. Multiple proteins might interfere with OA pathology, including tumor necrosis factor-alpha (TNFα)-stimulated protein 6 (TSG-6).

TSG-6, encoded by the TNFα-induced protein 6 (TNFAIP6) gene, is expressed by different cell types after inflammatory stimulation.[1] The protein consists of a Link and a CUB domain that can bind multiple proteins and extracellular matrix molecules[2] and has shown protective effects in experimental arthritis models. Transgenic mice with cartilage-specific constitutive overexpression of TSG-6 show chondroprotective effects of TSG-6 in the antigen-induced arthritis model.[3] In addition, recombinant TSG-6 also showed anti-inflammatory effects in collagen-induced arthritis.[1]

Several mechanisms have been proposed for the chondroprotective and anti-inflammatory effects of TSG-6.[2] Firstly, TSG-6 can activate the inter-α-inhibitor (IαI) by transferring its heavy chains to hyaluronan (HA). The activated IαI can subsequently inhibit the activation of plasmin and thus prevent the cleavage and activation of pro-matrix metalloproteinases (pro-MMPs). Secondly, TSG-6 can modulate the interaction between HA and CD44 to influence CD44-dependent migration of leukocytes into the joint.[4] In addition, TSG-6 has been shown to interact with CXCL8, BMP-2 and RANKL influencing inflammation and bone remodeling.[5,6]

Because cartilage damage, inflammation and bone remodeling have also been implicated in the pathogenesis of OA, we hypothesized that TSG-6 gene therapy might also have protective effects in experimental OA. In this study we first showed functionality of the TSG-6 gene therapy *in vitro* by inhibiting osteoclast activity. Subsequently, we tested the TSG-6 gene therapy in the collagenase-induced OA (CIOA). We did not observe therapeutic effects of TSG-6 overexpression on inflammation or cartilage damage. In contrast, we observed ectopic bone formation at the medial femur/tibia region of the joint.

Materials and methods

Virus production

Vector cloning is described in the supplementary methods. Lentiviral and adenoviral vector production were performed as described previously.[7,8]

Bone marrow-derived cell culture

Bone marrow-derived cells (BMDCs) of C57BL/6 mice were obtained from the femur and the tibia. Bone marrow cells were flushed from the bones using RPMI medium supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate and 1% penicillin/streptomycin (P/S) using a Microlance 3 needle (Becton Dickinson (BD), Breda, The Netherlands). Cells were passed trough a 70 µm cell strainer (Corning, NY, USA) and centrifuged for 5 min at 1500 rpm/423 g in a Heraeus Megafuge 16R (Thermo Scientific). In a 96-well plate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), 10^5 cells were seeded on the surface of the plate for differentiation analysis or on an elephant dentin slice for the bone resoption assay. Osteoclast differentiation was induced in alpha-MEM medium, supplemented with 5% FBS, 1% P/S, 30 ng/ml M-CSF (R&D systems, Oxford, UK) and 20 ng/ml RANKL (R&D systems). For tartrate resistant acid phosphatase (TRAP) staining, after 72h the the osteoclast medium was replaced to induce osteoclast fusion with or without 1 µg/ml recombinant murine TSG-6 (rmTSG-6) (R&D) for 24h. Subsequently, cells were fixed and stained with the TRAP staining kit (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's protocol. For the bone resorption assay, after 72h the differentiation medium was replaced and cells were transduced with 166 ng lentivirus per 10^5 cells in each well or left untreated until day 4, at which 1 µg/ml rmTSG-6 was added to the rmTSG-6 group to align with protein production in the TSG6-transduced cells. At day 7, medium was removed from all dentin slices and cells were lysed with H_2O.

Resorption assay

After incubation with the osteoclasts, dentin slices were incubated with 10% NH_3 and sonicated for 20 cycles 30 seconds on/off. After washing, the slices were incubated for 10 minutes with 10% $Kal(SO_4)_2$ and stained with PhastGel Blue R-350 coomassie tablets (GE Healthcare, Eindhoven, The Netherlands) according to the manufacturer's protocol. For the quantification, 5 pictures at 200x magnification were taken from every dentin slice using the Labovert FS (Leitz, Leica, Rijswijk, The Netherlands). The Leica application Suite (LAS) was used to analyse the average bone resorption percentage per dentin slice.

Synovial biopsy culture

Synovial biopsies were obtained from surplus C.B-17 mice after sacrifice. Using a 3 mm biopsy punch (Stiefel, Wachtersbach, Germany), synovial explants were obtained and combined from the lateral and medial synovium. The biopsies were kept in 200 µl RPMI medium supplemented with 1 mM pyruvate and 1% P/S and 10^7

infectious units adenovirus CMV-luc or CMV-TSG6 was added. After 2h, 20 µl FBS was added and after 24h, total RNA was isolated using 500 µl Tri reagent (Sigma) according to the manufacturer's protocol and processed and analyzed as decribed in the supplementary materials.

Collagenase-induced OA

Detailed animal experimental procedures are described in the supplementary methods in accordance with the ARRIVE guidelines. In short, 30 female C57Bl/6J mice (Janvier) received intra-articular (i.a.) injections of 10^7 infectious units adenovirus CMV-luc or CMV-TSG6 4 days prior to the start of the induction of the model in the right knee. Collagenase-induced OA (CIOA) was induced by two i.a. injections of 1 unit collagenase type VII (Sigma) at day 1 and day 3. A second virus injection was given at day 21 of CIOA and mice were sacrificed at day 42.All animal experiments were approved by the local authority Animal Care and Use Committee and local ethics committee (RU-DEC 2014-080).

Prosense measurement

6 days after the first collagenase injection, 5 mice per group received i.v. injections of 1,33 nmol Prosense 680 probes (PerkinElmer, Groningen, The Netherlands) in 100 µl in the orbita plexus. At day 7, hair was removed from the knees and the mice were imaged with the IVIS Lumina (PerkinElmer) using the Cy5.5 filter. The data was analyzed using Living Image 3.0 (PerkinElmer). Regions of interest (ROIs) of the same size were drawn around the knees and the florescence intensity were determined for the experimental and contralateral knees.

X-ray imaging

After sacrifice, the mouse knees were removed and imaged using the Faxitron FX-20 (Faxitron, Tucson, AZ, USA) at 26 KV for 10 seconds. The images were blinded and randomized and the ectopic bone formation was given an arbitrary score of 0-5. 0=no ectopic bone formation visible, 1=ectopic bone formation just detectable, 2=clear ectopic bone formation, 3=bone formation stretching along femur and tibia, 4=large ectopic bone formation, 5=severe ectopic bone formation, similar to the most severe sample.

Histological analysis

After X-ray imaging, knee joints were fixed in formalin, embedded in paraffin and cut in 7 µm sections. The sections were stained with Safranin-O/Fast Green,

blinded and randomized. Cartilage damage was assessed using an arbitrary score of 0-30 based on the modified OARSI cartilage OA histopathology grading system as described by Glasson et al. OAC 2010. Several mice showed dislocation of the knee joint, but these were distributed equally between the groups and are incorporated in the analysis.

Statistical analysis

Statistical comparisons were performed by One-way analysis of variance (ANOVA) and Mann-Whitney *U* test as indicated in the text using GraphPad Prism 5.03. Results are depicted as mean +/- 95% confidence interval (CI) and p-values below 0.05 were regarded as significant.

Results

We first tested the functionality of viral expression of TSG-6 *in vitro*. BMDCs were differentiated to multinucleated osteoclasts using M-CSF and RANKL in the absence or presence of recombinant murine TSG-6. We did not observe any effect of TSG-6 on the ability of the BMDCs to form multi-nucleated osteoclasts **(Fig. 1A)**. We subsequently assessed the effects of TSG-6 on osteoclast resorption activity on dentin slices. Under positive control conditions with lentiviral control virus, resorption pits covered ~20% of the dentin surface **(Fig. 1B)**. The resorption by the multinucleated osteoclasts was significantly reduced by both recombinant TSG-6 (~72%) and the lentiviral expression of TSG-6 (~49%). This shows that viral overexpression of TSG-6 can result in functional levels of TSG-6.

Overexpression of TSG-6 in synovium was first investigated in synovial explants. After transduction with adenoviral CMV-TSG6, the expression of TSG-6 was significantly increased compared to CMV-luciferase **(Fig. 2A)**. The protective effects of TSG-6 on inflammation and cartilage damage were tested in the CIOA model. Mice received an injection with adenovirus to provide high expression levels of TSG-6 or control luciferase in the right knee 4 days prior to the first collagenase injection[8]. The effects of TSG-6 on inflammation-associated protease activity was determined in a subset of the mice after injection of Prosense 680 probes at day 6 after the first collagense injection. At day 7, the fluorescence intensity was measured. No strong protease activity was measured compared to the naïve contralateral knee and no significant

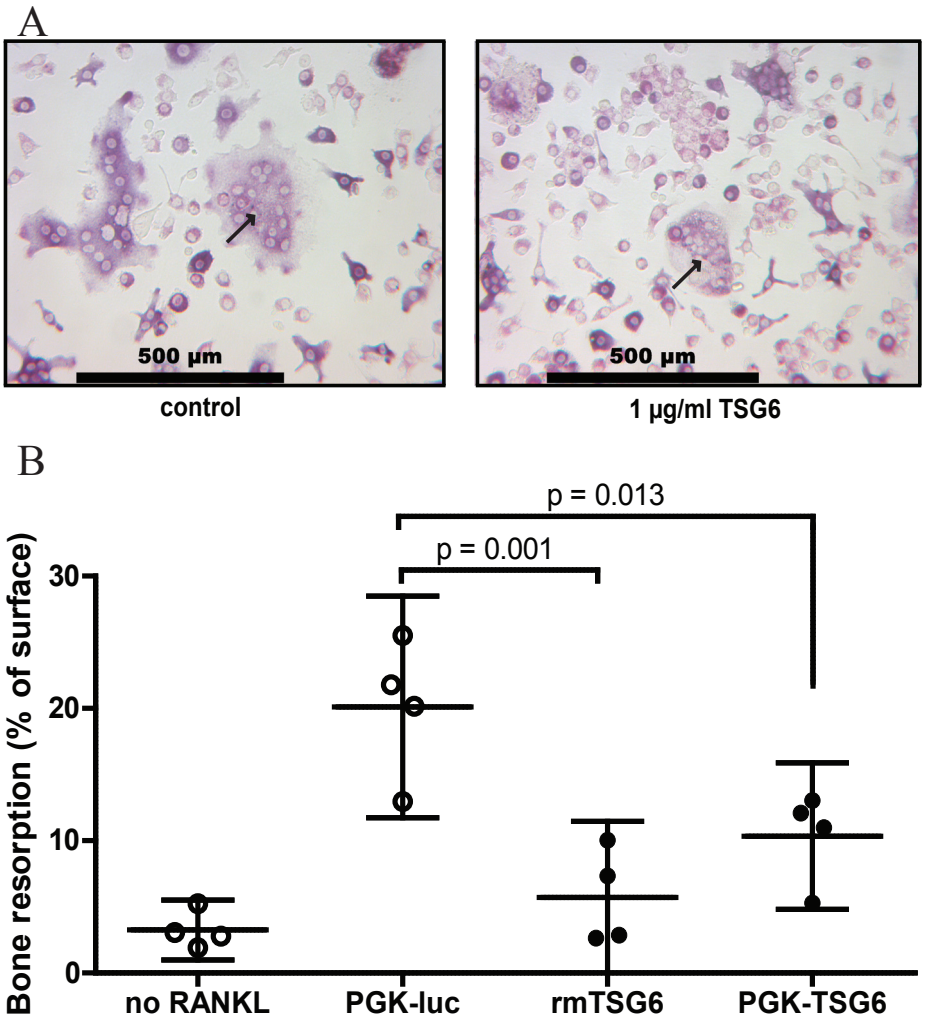
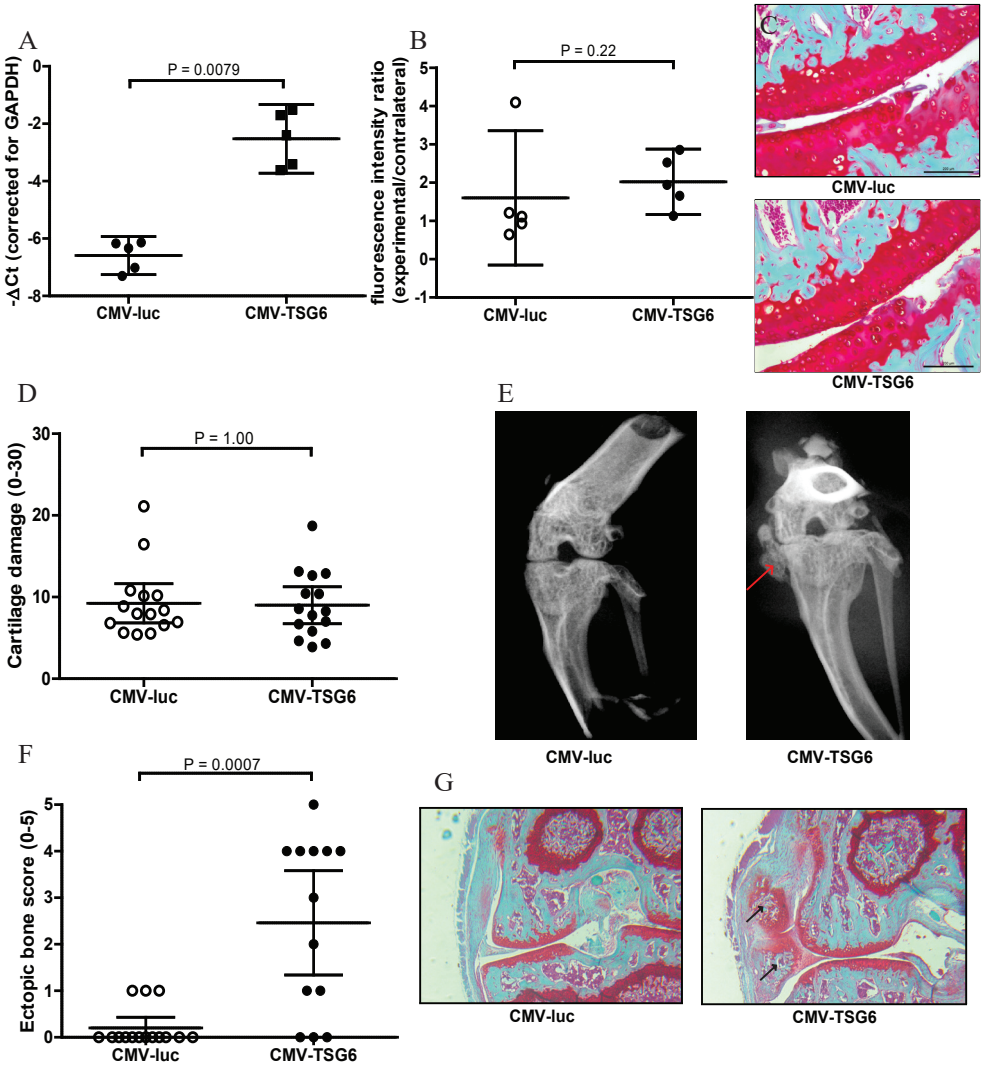


Figure 1: Osteoclastogenesis and bone resorption by bone marrow-derived cells (BMDCs) after TSG-6 treatment. (A) TRAP staining of BMDCs differentiated to osteoclasts using M-CSF and RANKL. Multi-nucleated osteoclasts are indicated by black arrows. **(B)** BMDCs were seeded on dentin slices and differentiated to osteoclasts. After 3 days of differentiation, cells were transduced with control of TSG-6 virus or not transduced. After 24 hours, 1 µg/ml recombinant murine TSG-6 (rmTSG6) was added to the rmTSG-6 group The cells were incubated for 3 additional days and thereafter the resorption pits were evaluated. RANKL was present during the whole experiment, but was omitted in the 'no RANKL' group. Every sample represents an average of 5 pictures and the bone resorption is depicted as percentage resorption of the complete surface. Statistical comparisons were performed by one-way ANOVA.

differences were found between the TSG-6 adenovirus and the luciferase control virus **(Fig. 2B)**. A second adenovirus injection was given at day 20 to provide TSG-6 expression for the second half of the model and mice were sacrificed at day 42. The cartilage damage was assessed in histological sections using the OARSI cartilage OA histopathology grading system. No significant differences were observed in cartilage damage between the control group and the TSG-6 treated group **(Fig. 2C,D)**.

Before the joints were processed for histological analysis, the bone structure was analysed using X-ray imaging and scored using an arbitrary scoring method from 0 to 5. Surprisingly, mice treated with adenoviral TSG-6 showed significantly more ectopic bone formation (77%) compared to the control group (20%) located at the medial collateral ligament **(Fig. 2E,F)**. Safranin-O staining of histological sections of the joints shown in Figure 2G show the presence of cartilage around the ectopic bone, indicating that the ectopic bone formation might be the result of cartilage ossification **(Fig. 2G)**.

Figure 2: Effects of adenoviral luciferase or TSG6 on collagenase-induced osteoarthritis (CIOA). **(A)** Expression of TSG-6 in synovial explants, 24h after transduction with adenoviral CMV-luciferase (CMV-luc) or CMV-TSG6. **(B)** Prosense measurement at day 7 to assess the inflammation-associated protease activity. The fluorescence intensity ratio compared to the contralateral knee was calculated. **(C)** Magnification of examples of cartilage damage. Representative pictures of the medial femur and tibia are shown after Saphranin-O staining. **(D)** Cartilage damage at day 42. For every knee joint, the cartilage damage was determined in the medal tibia, medial femur, lateral tibia and lateral femur in three sections. The average cartilage damage is depicted. **(E)** Typical X-ray images of knee joints at 42. Ectopic bone formation is indicated by the red arrow. **(F)** Arbitrary scoring of ectopic bone formation (0-5) in the X-ray images. **(G)** Histological sections of the knee joints shown in Figure 3E. Sections were stained with Safranin-O and counterstained with Fast Green. Ectopic bone formation is indicated with black arrows. Statistical comparisons were performed by Mann-Whitney *U* test.



Discussion

In this study, we show that *in vitro* viral overexpression of TSG-6 in BMDCs has no effect on multinuclear osteoclast formation, but can reduce resorption activity of the osteoclasts. This has been observed in earlier studies[6] and shows that functional TSG-6 can be expressed after viral gene transfer. However, when tested in the CIOA model, TSG-6 could not reduce cartilage damage. In contrast, we observed increased ectopic bone formation in mice with TSG-6 overexpression.

Although TSG-6 is associated with different functions that might be protective for OA, the expression and activity of TSG-6 correlate with progression of OA.[9] The exact mechanisms by which TSG-6 might be involved OA progression is not completely understood and is difficult to study because of the ability of TSG-6 to bind many different proteins and matrix components. One potential mechanism could be related to a disturbance of damage repair mechanisms. TSG-6 has been shown to bind to fibronectin (FN), stimulating FN matrix assembly involved in damage repair.[10] However, TSG-6 can also serve as a bridging molecule between FN and thrombospondin-1 (TSP-1). TSP-1 is increased in OA chondrocytes and osteophytes and has multiple functions, including the activation of latent Transforming Growth Factor-β (TGF-β).[11,12] Increased TGF-β activity has previously been correlated with chondrocyte differentiaton and enthesophyte formation similar to this study in the medial collateral ligament in CIOA.[8,13]

An alternative mechanism for increased TGF-β activity could be related to HA. In the context of fibrosis, studies have shown that the heavy chain transfer to HA by TSG-6 can stabilize the HA coat, which is essential for TGF-β-mediated myofibroblast differentiation.[14] Possibly, TSG-6 can increase TGF-β-mediated chondrogenesis in a similar way. HA could also increase chondrogenesis in adipose-derived stem cells.[15] This was dependent on the interaction between HA and CD44, which is influenced by TSG-6.[4] The formation of bone is dependent on the ratio between bone matrix deposition and bone resorption. The inhibiting effects on osteoclast activity observed in Figure 1B might favor the anabolic activity and stimulate the ectopic bone formation. Although the exact mechanism by which TSG-6 can increase ectopic bone formation *in vivo* remains to be elucidated, no improvement in cartilage damage in CIOA was observed, indicating that intra-articular gene therapy with TSG-6 might not be a promising treatment for OA and our study places TSG6 as a potential target in OA that warrants future studies.

Funding source

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Ethical approval of animal studies

All animal experiments were approved by the local authority Animal Care and Use Committee and local ethics committee (RU-DEC 2014-080). Animal care was in accordance with the institution guidelines.

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Supplementary methods

Vector cloning

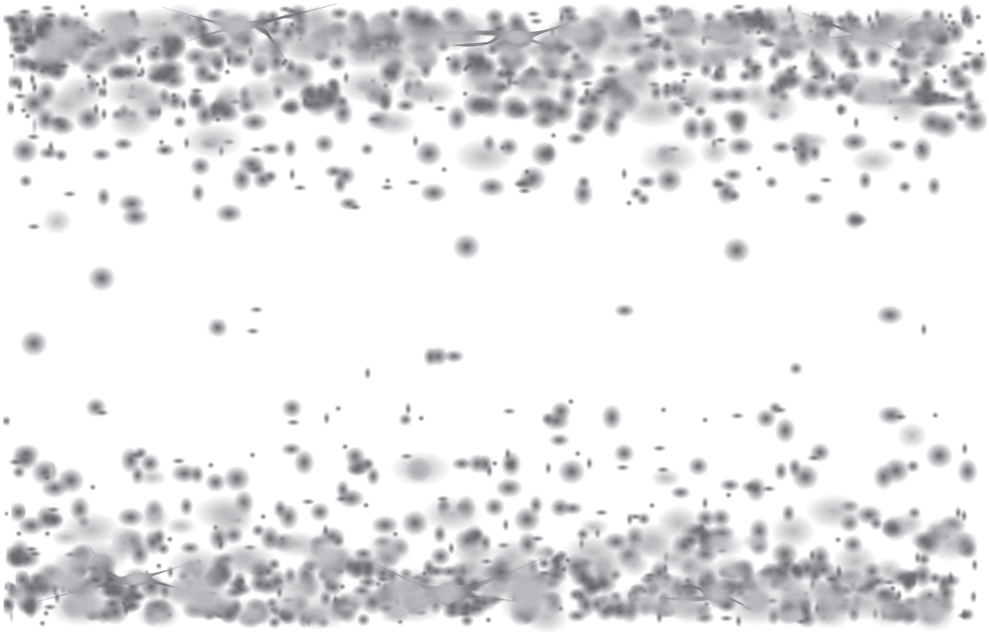
For cloning of the third generation self-inactivating lentiviral vector (sin), the TSG-6 coding sequence was obtained from a cDNA sample of inflamed synovium of a C57BL/6 mouse by nested PCR. The first round of PCR was done by amplification using forward primer 5'-CGGCTCTGCAACCGAAGA-3' and reverse primer 5'-ATCCAAAAGTATTTATTACAGCAAT-3'. For the second round of amplification, forward primer 5'-GTCGACGCCACCATGGTCGTCCTCC-3' and reverse primer 5'-CATATGATCCAAAAGTATTTATTAC-3' were used, introducing restriction sites for SalI and NdeI respectively. The TSG-6 gene was cloned in the pRRL-cPPT-PGK-luc-PRE-SIN vector using the strategy previously described[1]. As a control vector, the Trifusion reporter gene was digested from the pcDNA3.1 vector (kind gift from Robert E. Reeves, Stanford University) using NheI and XbaI (New England Biolabs). For adenovirus cloning, TSG-6 was digested from the PCR-script CAM vector using SalI and ligated in the SalI pre-digested pShuttle-CMV (Stratagene, La Jolla, CA, USA). The CMV-luciferase adenovirus was used as control vector.

RNA isolation and qPCR

Synovial biopsies in Trizol were first homogenated using Magnalyzer Green Beads (Roche Life Sciences, Almere, The Netherlands) and processed according to the provided protocol. DNA was removed by DNAse and cDNA was generated using Moloney murine leukemia virus reverse transcriptase, 0.5µg/µl oligo(dT) primers and 12.5mM dNTPs (Thermo Scientific). Quantative real-time PCR was perfomed as previously described[7] using forward primer 5'-CAACCCACATGCAAAGGAG-3' and reverse primer 5'-TACTCATTTGGGAAGCCCG-3'.

Collagenase-induced OA

30 Female C57BL/6J mice (Janvier) of 10-12 weeks old were randomized and housed in groups of 5 in filtertop cages at DM-II level with 12-h light-dark cycles and water and standard diet (AB Diets, Woerden, The Netherlands) were provided ad libitum. The mice received an intra-articular (i.a.) injection of 10^7 infectious units adenovirus CMV-luc or CMV-TSG6 dissolved in 6 µl 0.9% NaCl 4 days prior to the start of the induction of the model. All i.a. injections were performed during the day in the right knee using a BD microlance needle 30G 1/2' (BD) under general anaesthesia using 2,5% isoflurane. The injections were performed by a technician without knowledge of the viral vector content in a laminar flow cabinet. Collagenase-induced OA (CiOA) was induced by two i.a. injection of 1 unit collagenase type VII (Sigma) in 6 µl 0.9% NaCl at day 1 and day 3. At day 21, a second injection with the same viral vector was given and mice were sacrificed by cervical dislocation at day 42.



Chapter 4

Functional Tissue Analysis Reveals Successful Cryopreservation of Human Osteoarthritic Synovium

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Osteoarthritis (OA) is a degenerative joint disease affecting cartilage and is the most common form of arthritis worldwide. One third of OA patients have severe synovitis and less than 10% have no evidence of synovitis. Moreover, synovitis is predictive for more severe disease progression. This offers a target for therapy but more research on the pathophysiological processes in the synovial tissue of these patients is needed. Functional studies performed with synovial tissue will be more approachable when this material, that becomes available by joint replacement surgery, can be stored for later use. We set out to determine the consequences of slow-freezing of human OA synovial tissue. Therefore, we validated a method that can be applied in every routine laboratory and performed a comparative study of five cryoprotective agent (CPA) solutions. To determine possible deleterious cryopreservation-thaw effects on viability, the synovial tissue architecture, metabolic activity, RNA quality, expression of cryopreservation associated stress genes, and expression of OA characteristic disease genes was studied. Furthermore, the biological activity of the cryopreserved tissue was determined by measuring cytokine secretion induced by the TLR ligands lipopolysaccharides and Pam3Cys. Compared to non frozen synovium, no difference in cell and tissue morphology could be identified in the conditions using the CS10, standard and CryoSFM CPA solution for cryopreservation. However, we observed significantly lower preservation of tissue morphology with the Biofreeze and CS2 media. The other viability assays showed trends in the same direction but were not sensitive enough to detect significant differences between conditions. In all assays tested a clearly lower viability was detected in the condition in which synovium was frozen without CPA solution. This detailed analysis showed that OA synovial tissue explants can be cryopreserved while maintaining the morphology, viability and phenotypical response after thawing, offering enhanced opportunities for human *in vitro* studies.

Introduction

Osteoarthritis is a common joint disease characterized by degenerative alterations in the articular cartilage. However, it is becoming more evident that OA is not just a wear and tear disease of the cartilage but involves multiple joint tissues including the synovium.[1,2] The synovium is the tissue lining the joint capsule and in OA often shows signs of inflammation, for example by macrophages infiltrating the synovium. These macrophages produce pro-inflammatory cytokines like IL1β and TNFα, activating the fibroblast-like synoviocytes to release additional cytokines (IL6 , IL8) and matrix-degrading enzymes.[3] These cytokines and enzymes damage the articular cartilage, resulting in the release of damage-associated molecular patterns (DAMPS) that have pro-inflammatory properties as well, creating a perpetuating loop causing low grade chronic inflammation.[3,4] This inflammation is suggested to contribute to the disease phenotype and disease progression.[1,2,5] Studying the fundamental pathological processes in the OA synovium would help in finding a treatment for this disease.

Of importance for such studies is the availability of synovial tissue. Patient material becomes available as remnant material after joint replacement surgery, or via synovial biopsy. Functional studies on synovial tissue are often limited to a small number of patients as material needs to be used immediately when becoming available. Thus, timing is an important issue, hampering optimal planning and synchronization of an experiment. A solution would be to cryopreserve the valuable synovial tissue in such a way that the thawed synovial tissue performs identical to freshly obtained material. To our knowledge there are no studies describing cryopreservation of human OA synovial tissue in which the disease characteristic cell functions and interactions are maintained.

Although cryopreservation of cell lines is widely and successfully applied in almost every cell culture lab, the cryopreservation of primary cells and especially complex tissues is still challenging,[6-11] as no regeneration takes place. Furthermore, a tissue is composed of a mixture of cell types, all with their specific optimal cryopreservation requirements.[8,10] Size is another obstacle, as this influences CPA solution penetration and leads to differences in exposure to CPAs.[12] Globally, two cryopreservation methods exist, slow freezing and vitrification. Vitrification has theoretical advantages over slow freezing once the optimal conditions are determined. However, the high CPA concentration needed to vitrify the material is a major drawback, due to their toxicity.[10] Slow freezing uses low concentrations of CPA, reducing CPA toxicity. Another advantage of slow

freezing is that the procedure to get the tissue 'freezer-ready' is fast and easy to apply in every routine laboratory without needing advanced equipment.

We have compared four commercially available and one homemade CPA solution for their suitability to cryopreserve human OA synovial tissue. These CPA solutions are developed for application in a slow freezing protocol. To validate our method we studied the viability of the cells and the conservation of tissue-specific responses to inflammatory stimuli. Therefore, we studied the ability of the tissue to respond to the pathogen associated molecular pattern (PAMP) TLR ligands lipopolysaccharides (LPS) and Pam3Cys (P3C) by measuring cytokine secretion. Both DAMPs and PAMPs can activate macrophages and fibroblasts via the TLR-4 and TLR-2 pathway. TLR4 can be activated in similar fashion by the PAMP LPS and the OA related DAMP S100A8/9. TLR2 can be activated by the PAMP P3C and the OA related DAMP hyaluronic acid.[13] In previous studies PAMPs have been shown to upregulate several plasma proteins in the OA synovial fluid, similar to the DAMPs.[13] In this study we therefore included LPS and P3C to study the TLR-2/4 response.

This detailed analysis showed that OA synovial tissue explants can be cryopreserved while maintaining the morphology, viability and phenotypical response after thawing.

Materials & Methods

Synovial tissue

Synovial tissue was obtained as remnant material, after written informed consent, from OA patients undergoing joint replacement surgery at the Orthopedics department of the Radboud University Medical Center. Protocols were approved by the local committee on research involving human subjects (CMO region Arnhem-Nijmegen, the Netherlands) under NL-number 54055.091.15. Dissected synovial samples were immediately placed in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 1% penicillin–streptomycin at 4°C. To confirm synovial origin, characterized by the presence of a synovial lining, representative tissue samples were embedded in OCT and 6 µM cryosections were cut and subsequently stained by Hematoxilin and Eosin (H&E). Histology and general viability assays were performed with synovial tissue from three donors, gene expression analysis and cytokine secretion assays were performed with synovial tissue from four different donors. For microarray analysis synovial samples were obtained by surgery or via fine-needle arthroscopy from 10 OA patients and 7 controls.

Cryopreservation procedure

Biopsies were punched out of the synovium using a disposable 3 mm biopsy punch (KAI medical) and collected in RPMI medium (Gibco) containing 10% FCS, pyruvate and pen/strep. Biopsies were transferred into a cryovial and 1 ml of CPA solution (4°) was added. Five CPA solutions were compared in this study: 1. CS2 (BioLife Solutions), 2. CS10 (BioLife Solutions), 3. Standard: 10 % DMSO, 10% FCS in RPMI, 4. Cryo-SFM (PromoCell), 5.Biofreeze (Biochrom, Merck Millipore). The cryovial was incubated for 10 minutes on ice before being transferred to an isopropanol container (Nalgene, Mr. Frosty). The next day, the cryovials were relocated to liquid nitrogen where they were stored for one week. Thawing was performed by transferring the cryovial as quickly as possible from the liquid nitrogen into a 37°C water bath until all ice has disappeared. Subsequently, the tissue was washed 3 times 10 minutes in pre-warmed RPMI (37°C) before being cultured in RPMI (10% FCS, pyruvate and pen/strep) at 5% CO_2, 37°.

Histology and histological analysis

Non-frozen or thawed synovial biopsies from three patients were cultured for 24 hours before fixation in 4% formalin for 1 hour. For every condition, a minimum of 3 separate 3 mm biopsies was included. Fixation was followed by dehydration and embedding in paraffin. Sections were cut at 7 µm and stained by H&E. Histology of every biopsy was scored in duplo using an arbitrary scale (0-1) for global morphology, appearance of the intimal lining layer, cellular and nuclear morphology, morphology of infiltrating cells, blood vessel morphology and fat cell morphology. All parameters were given a score of 1, 0.5 or 0. A score of 1 indicated no observed change, a score of 0,5 was given when the morphology was clearly distinct from non-frozen and a score of 0 was given to complete structural loss and cell death of the tissue. The mean score of these parameters is displayed in Figure 1. Pictures were taken at 400x magnification.

General viability assays

Non-frozen or thawed synovial biopsies were cultured overnight in a 96-wells plate in 100 µl RPMI (10% FCS, pyruvate and pen/strep) as described above. For the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, 50 µl XTT reagent (Cell Proliferation Kit II, Roche) was added per well and the absorbance was measured at 450 and 655 nm after 2, 4 and 6 hours using a BioRad iMark microplate reader. Values were corrected for the reference wavelength and background signal. For the adenosine triphosphate (ATP) assay, 100 µl lysis

buffer (Promega, Cell-Titer Glo) was added to each well and vigorously shaken for 5 minutes before incubation for 25 minutes at room temperature. Luminescence was determined using the BMG Labtech CLARIOstar microplate reader. Values were corrected for the background signal and depicted as relative light units (RLU).

RNA isolation

Non-frozen and thawed synovial biopsies were cultured for 24 hours and subsequently snap-frozen in liquid nitrogen. Total RNA was isolated using the RNeasy fibrous tissue kit (Qiagen, Venlo, The Netherlands). Frozen synovial samples were transferred to MagNA Lyser Green Beads tubes (Roche) containing RLT lysis buffer. The tissue was disrupted in the MagNA lyser for 3 x 20 sec at 6000 rpm with one minute cooling between cycles. The remainder of the procedure was performed according to manufacturer instructions with on column DNA digestion.

RNA integrity assessments

The RNA integrity number (RIN) was determined directly after RNA isolation with the Agilent RNA 6000 Nano kit using the protocol provided by the manufacturer. The Nano chip was run on the Agilent 2100 Bioanalyzer using the 2100 Expert Software.

Microarray analysis

Microarray analysis was performed as described earlier.[14] Briefly, total RNA was isolated from the synovial samples, using the RNeasy kit for fibrous tissues (Qiagen, Venlo, The Netherlands). Total RNA (100 ng) was hybridized to a U133Plus 2.0 oligonucleotide array (Affymetrix, Santa Clara, CA). The arrays were scanned with a GeneChip scanner (Affymetrix) and analyzed with GeneChip operating software version 1.4 (Affymetrix). Array normalization and expression value calculations were performed with DNA-Chip Analyzer version 1.3 (www.dchip.org), using the invariant set normalization and the model-based method.[15] The OA synovial samples and control samples were deposited in the GEO database under accession number GSE82107.

qPCR analysis

An unpublished systematic review of the literature was performed to find genes upregulated during cryopreservative stress. All Pubmed and Embase articles depicting cryopreservation and gene expression analysis of living human and animal cells and tissue were assessed and revealed six genes of which the expression is known to respond to cryopreservation conditions: two heat shock protein genes

(*HSPA1A,*[16] *HSP27*[17]*)* and four apoptosis-related genes (*CASP3,*[16] *BAX,*[18] *CD95* [16] *and MCL1*[16]). The expression of above mentioned genes was either increased or decreased (*HSPA1A*, *MCL1*) in comparison to non frozen cells or tissue. We determined their expression level after 24 hour culture.cDNA synthesis and qPCR analysis were performed as described previously.[14] Primer sequences are listed in Table S1.

Multiplex ELISA

Non-frozen and thawed synovial tissue was cultured for 24 hours in the presence or absence of LPS (100 ng/ml) and Pam3CSK4 (P3C) (1 μg/ml). Cytokine concentrations (IL1β, TNFα, IL-6 and IL-8) were determined in culture supernatants by Luminex multianalyte technology using the Bio-Plex 200 (Bio-Rad, Hercules, CA) and the Bio-Plex pro human cytokine kits (Bio-Rad) according to manufacturer instructions. To measure IL-6 and IL-8 levels, culture supernatants were diluted 200-fold.

Statistics

For statistical analysis GraphPad Prism version 5.03 was used. To determine significant differences between groups ANOVA was performed in combination with the Tukey test (Fig 1-5). To determine significant differences between non-stimulated and stimulated tissue for cytokine expression, the Student t-test was applied (Fig 6). Results are depicted as means +/- SD, and p values below 0.05 were regarded as significant.

Results

Effect of cryopreservation on tissue morphology and cell composition

We first determined the effects of cryopreservation on tissue morphology and histological integrity by comparing freezing with five different cryopreservation media and freezing without cryoprotectant to non-frozen biopsies. The histological tissue integrity was assessed for structural integrity of the different structures within the tissue and cell death using an arbitrary scoring system.A significantly lower histological score was found for the CS2, Biofreeze and without CPA condition compared to the non-frozen condition **(Fig. 1A-F)**. When studying the individual patients highest differences were observed in patient 1. A relatively high cellularity and influx of inflammatory cells were present in the tissue of patient 1 **(Fig. 1B)**, which were not observed in the tissue of the second patient **(Fig. 1C)**, indicating that

infiltrating cells might be more vulnerable to cryopreservation-induced damage. This was in line with the observation that the lowest histological scores were obtained for the parameter 'morphology of infiltrating cells' using the CS2 (patient 1: 0.4, p2: no infiltrating cells present, p3: 0.5) and Biofreeze medium (patient 1: 0.56, p2: no infiltrating cells present, p3: 0).

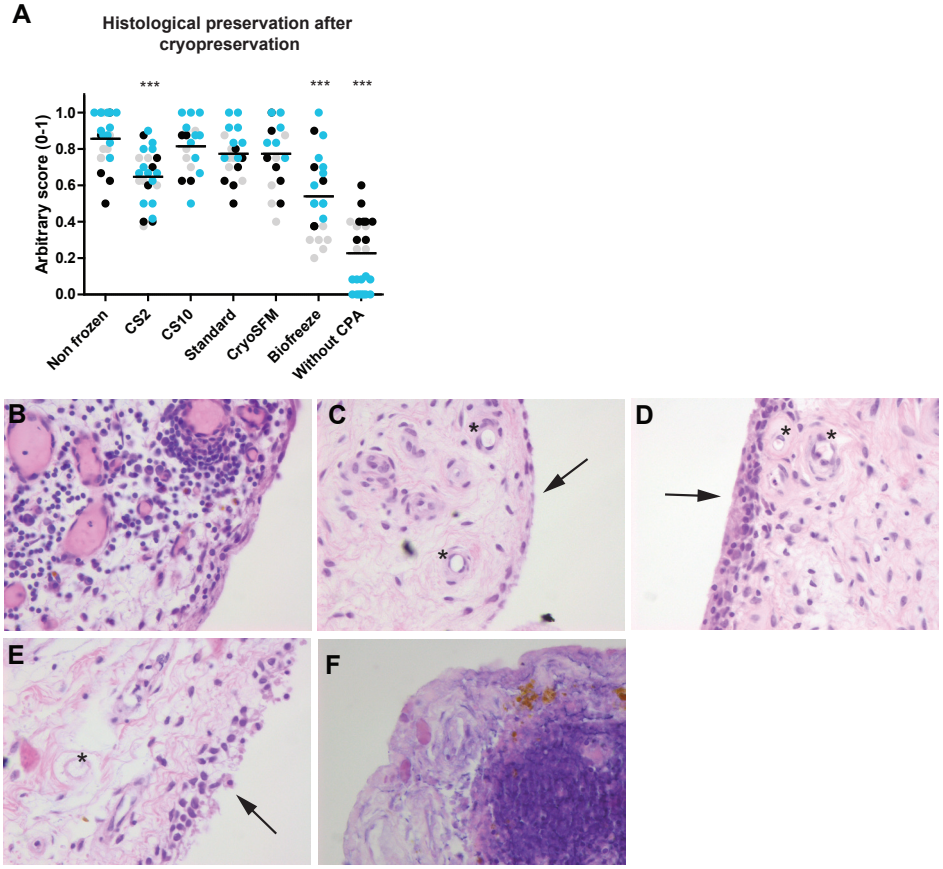

Figure 1. Histological analysis of cryopreserved synovial explants.

(A) Histological preservation was determined by assessing global histological parameters using an arbitrary score. Mean score is displayed. A significantly lower score was determined for the CS2, Biofreeze and without CPA condition. Blue dots: patient 1, black dots: patient 2, grey dots: patient 3. For statistical analysis, one-way ANOVA was performed, comparing the non frozen control to the cryopreserved conditions. *** $p<0.001$. Displayed is the mean. **(B)** Non frozen highly cellular synovium from patient 1. **(C)** Synovium of low cellularity cryopreserved with CryoSFM of patient 2 displaying an intact and smooth intimal lining layer (arrow), intact cells and blood vessels (*). **(D)** Synovium from patient 3 cryopreserved with CryoSFM, displaying mainly intimal lining hyperplasia (arrow), the

lining is smooth and intact, cells and blood vessels (*) are intact. **(E)** Synovium of patient 2 cryopreserved using the Biofreeze medium, showing a disrupted intimal lining layer (arrow), a high number of disrupted cells and disrupted blood vessels (*). **(F)** Synovium of patient 1 frozen without protecting CPA solution showing almost complete disruption of cells.

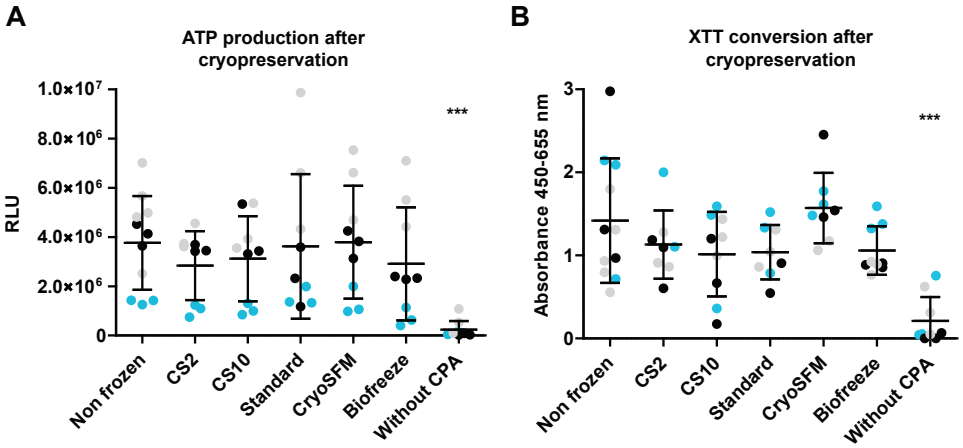

Figure 2. No change in metabolic activity in synovial explants after thawing. ATP and XTT levels were determined in triplo in OA synovial tissue of 3 patients. Patient 4: blue dots, patient 5: black dots, patient 1: grey dots. **(A)** Shown are the relative light units (RLU) **(B)** Shown is the percentage increase in absorbance over a 4 hour period, depicted as the mean +/- the SD. Statistical analysis was performed by one-way ANOVA, comparing the non-frozen control to the cryopreserved conditions. *** $p<0.001$.

Effect of cryopreservation on tissue viability

The metabolic activity reflects the global cellular viability of the tissue. The effect of cryopreservation on the metabolic activity of human OA synovial tissue from three patients was measured using the ATP and XTT metabolic assays **(Fig. 2)**. Both assays showed similar results: all conditions displayed a lower, though not significant decrease in metabolic activity compared to the non-frozen control and the condition cryopreserved without CPA solution showed a significant decrease in both assays.

Effect of cryopreservation on RNA integrity

Cell death leads to rapid RNA degradation, so the RNA quality (RIN values) is a direct measure of cryodamage and low RIN values impair reliable gene expression

analysis by microarray analysis. This analysis was performed to check whether the cryopreserved synovium is suitable for gene expression analysis by microarray studies, requiring minimal RIN values of 7. As depicted in figure 3, high RIN values, well above 7, could be obtained for the non-frozen and cryopreserved tissues. No significant differences could be detected between the non-frozen condition and the cryopreserved conditions. However, not every tissue biopsy yielded enough RNA (> 5 ng/ul) to successfully determine the RIN. This was the case in the biopsies that were cryopreserved without protecting CPA solution. This might indicate that the amount of isolated RNA gives an indication of the number of intact cells in the tissue at the time of isolation. As depicted in figure 3 the non-frozen, CS10 and CryoSFM conditions have a higher number of biopsies with an appropriate yield than the CS2, Standard and Biofreeze conditions. Furthermore, the average RIN value of the CryoSFM condition was the highest, although not significant and displayed the smallest standard deviation of all conditions tested **(Fig. 3)**. These results indicate that cryopreserved tissue can be used for gene expression analysis by qPCR. However, for microarray analysis several 3 mm biopsies might need to be pooled to obtain a sufficient amount of RNA.

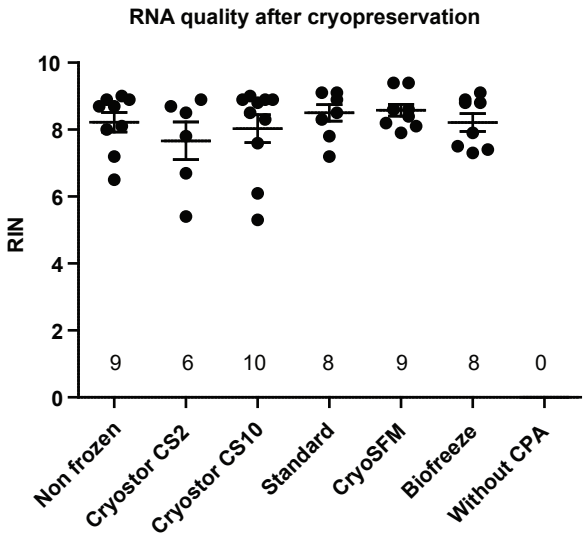

Figure 3. No effect of cryopreservation on RNA integrity in synovial explants. The RIN was determined in triplo of OA synovial tissue from four patients, 24 hours after thawing. Numbers indicate the number of biopsies of which the RIN could be successfully determined. Displayed is the mean +/- the SD. Statistical analysis was performed by one-way ANOVA, comparing the non-frozen control to the cryopreserved conditions.

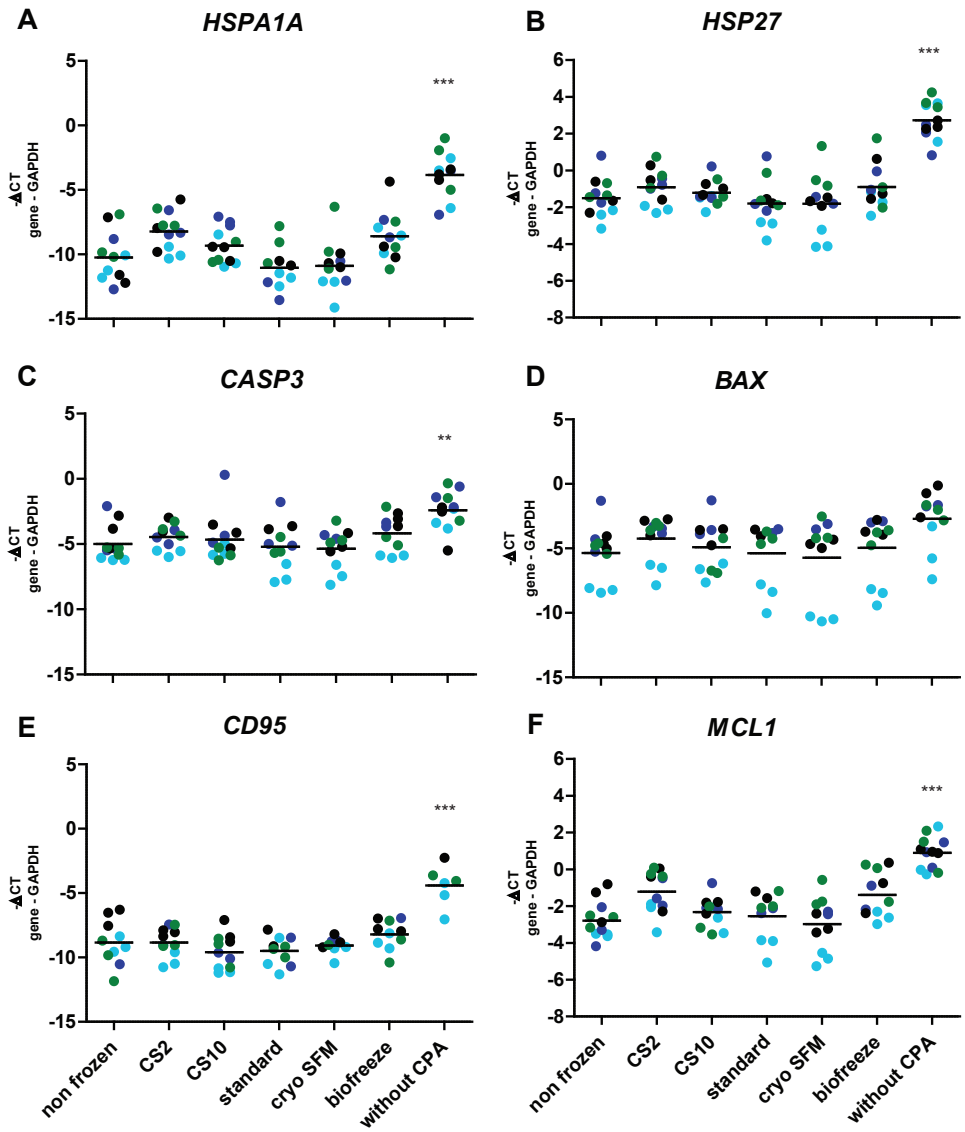

Figure 4. No increase in stress gene expression after cryopreservation. Gene expression of **(A)** *HSPA1a,* **(B)** *HSP27,* **(C)** *CASP3,* (**D**) *BAX,* **(E)** *CD95* and **(F)** *MCL1* was determined by qPCR analysis in four patients, 24 hours after thawing. Patient 6: green dots, patient 7: black dots, patient 8: dark blue dots, patient 4: light blue dots. Displayed is the mean. Statistical analysis was performed by one-way ANOVA, comparing the non-frozen control to the cryopreserved conditions. ** p<0.01; *** p<0.001.

Effect of cryopreservation on stress gene expression

We compared the expression of 2 heat-shock proteins and 4 apoptosis-related genes in response to cryopreservation and CPA solutions. Compared to the non frozen control no differences could be detected in the cryopreserved conditions **(Fig. 4A-F)**. Higher levels of stress gene expression were found in the negative control condition where the tissue was cryopreserved without protecting CPA solution). When studying the individual patients no other significant differences could be observed than already described above, except for the *MCL1* gene in patient 6. Here, a significant upregulation was identified for the CS2 and Biofreeze condition **(Fig. 4F)**. These results show that cryopreservation of OA synovial tissue does not induce upregulation of the selected stress genes indicating the absence of a stress response in the OA synovial tissue 24 hours after thawing.

Effect of cryopreservation on disease characteristic gene expression

Analyzing tissue morphology, metabolic assays and stress gene expression does not reflect the interaction between cell types, typical for proper tissue functioning. Therefore, we have functionally analyzed the tissue by determining the expression of disease-characteristic genes.

Disease-characteristic genes were selected via microarray analysis performed on synovial samples from 10 OA patients and 7 control probands. *TIMP3*, *CCL18* and *MMP9* were highly expressed in all patients and showed a fold increase of 14.1, 9.11 and 7.56 respectively compared to the control samples, indicating that these genes are characteristic for the OA disease phenotype (data not shown). We analyzed in four patients whether the expression of these three genes would be influenced by cryopreservation. Cryopreservation did not influence the expression of these three genes as no significant changes could be observed compared to the non frozen control **(Fig 5A-C)**. When patients were analyzed separately significant differences could be observed (Table 1). The Biofreeze CPA solution showed the most changes in gene expression. Furthermore, most changes in gene expression were observed in biopsies from patient 7 while none were identified in patient 8, indicating differences in tissue sensitivity to cryopreservation between patients **(Table 1)**.

Table 1. Analysis of cryopreservation-induced changes in disease- characteristic gene expression compared to non-frozen tissue in individual OA patients.

Expression of TIMP3, CCL18 and MMP9 was determined in four patients (P6-P8, P4, Figure 5). Unchanged refers to no changes in gene expression compared to the non frozen condition. Where a CPA solution is mentioned an arrow indicates whether the expression of the gene was upregulated or

down regulated compared to non frozen tissue. Statistical analysis was performed by one-way ANOVA, comparing the non frozen control to the cryopreserved conditions. *p<0.05; **p<0.01.

Symbol	Gene	P6	P7	P8	P4
TIMP3	TIMP metallo-peptidase inhibitor 3	unchanged	CS2 ↑ ** Standard ↑ * Biofreeze ↑ **	unchanged	unchanged
CCL18	chemokine (C-C motif) ligand 18	unchanged	Biofreeze↓ **	unchanged	Biofreeze ↓ **
MMP9	matrix metallo-peptidase 9	CS2 ↑ ** CS10 ↑ *	unchanged	unchanged	unchanged

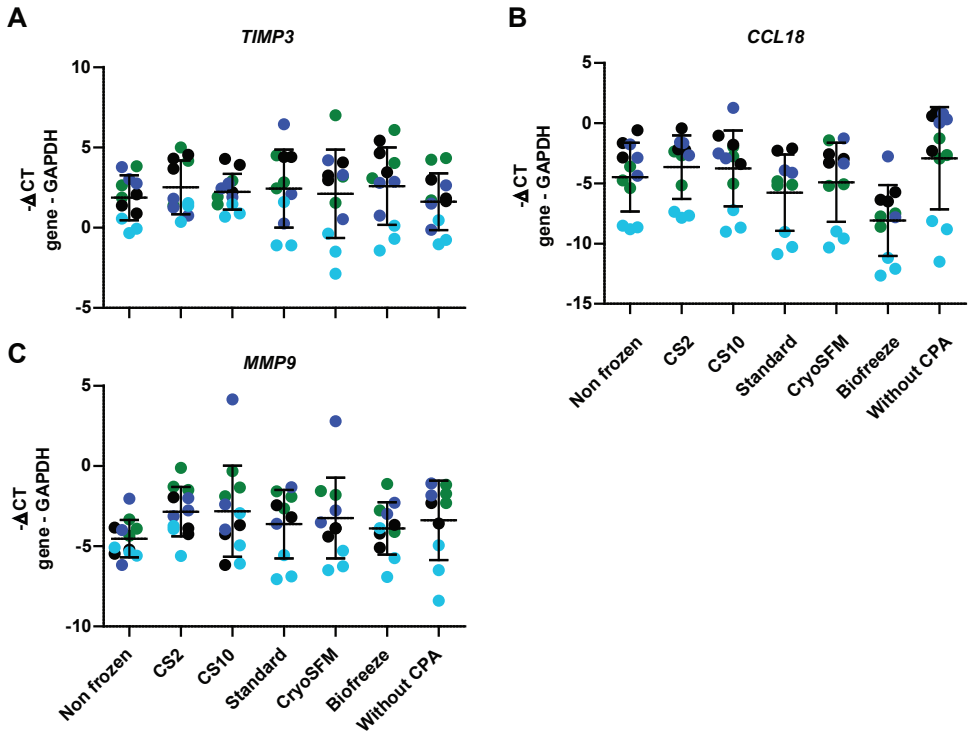

Figure 5. No effect of cryopreservation on disease characteristic gene expression.

Gene expression of *TIMP3* **(A)**, *CCL18* **(B)** and *MMP9* **(C)** was determined after thawing and 24 hours culture of synovial biopsies of four patients. Per patient 3 biopsies were included in the analysis. Patient 6: green dots, patient 7: black dots, patient 8: dark blue dots, patient 4: light blue dots. Displayed is the mean +/- SD. Statistical analysis was performed by one-way ANOVA, comparing the non-frozen control to the cryopreserved conditions.

Effect of cryopreservation on cytokine secretion

To analyze whether the inflammatory pathways, involved in the pathology of part of the OA patients, were affected by cryopreservation, we studied the spontaneous release of TNFα, IL1β, IL6 and IL8 into the culture medium **(Fig. 6A-D)**. For IL6, a significantly lower cytokine secretion was only observed compared to the non-frozen control in the condition without CPA **(Fig. 6C)**. Although, compared to the non frozen control, clear decreases were observed for the other cytokines in the condition without CPA, these were not significant. However, no changes were observed in all conditions cryopreserved with a CPA solution compared to the non frozen control condition. When studying the individual patients (not shown) significant differences were detected for patient 7 for IL6 secretion which showed lower levels in the CS2 (228 ± 115 ng/ml), CS10 (378 ± 53 ng/ml) and CryoSFM (312 ± 83 ng/ml) condition compared to the non frozen control (1182 ± 626 ng/ml). For patient 4 TNF secretion was higher in the Biofreeze condition compared to the non frozen control (38 ± 6 pg/ml vs 18 ± 8 pg/ml).

The intrinsic capacity of synovial tissue to respond to TLR-ligands P3C and LPS by cytokine release was also tested after cryopreservation. Again, only for IL6 a significantly lower cytokine secretion was observed compared to the non frozen control in the condition without CPA while not for the other conditions cryopreserved with a CPA solution, nor for IL1β, TNFα and IL8 in the condition cryopreserved without CPA **(Fig 6)**. Although a clear decrease compared to the non-frozen control was observed in the secretion of these cytokines as well. At the individual patient level the IL8 secretion was significantly decreased in patient 7 (70 ± 67 ng/ml vs 1619 ± 49 ng/ml) and 8 (8 ± 2 ng/ml vs 1358 ± 639 ng/ml) in the condition without CPA compared to the non frozen control. Furthermore, compared to the non frozen control, the IL6 secretion was decreased in the Biofreeze condition in patient 4 (536 ± 396 ng/ml vs 2430 ± 1062 ng/ml).

For every condition a clear increase in cytokine secretion was observed after stimulation, indicating the tissue is able to respond to inflammatory stimuli. However, the increase in IL1β secretion was not significant for the condition in which the synovium was cryopreserved in the standard medium and without CPA (p-values for all conditions are shown in Table S2). For IL6 the increase was not significant for the Biofreeze and without protection condition and for IL8 the increase was not significantly different for the CS2 medium and without protection condition. These results indicated that except for TNF secretion, the tissue was not capable of responding to inflammatory stimuli after being cryopreserved without any protecting CPA solution. After cryopreservation with the CS10 and CryoSFM

medium the tissue showed upregulation of all four cytokines studied.

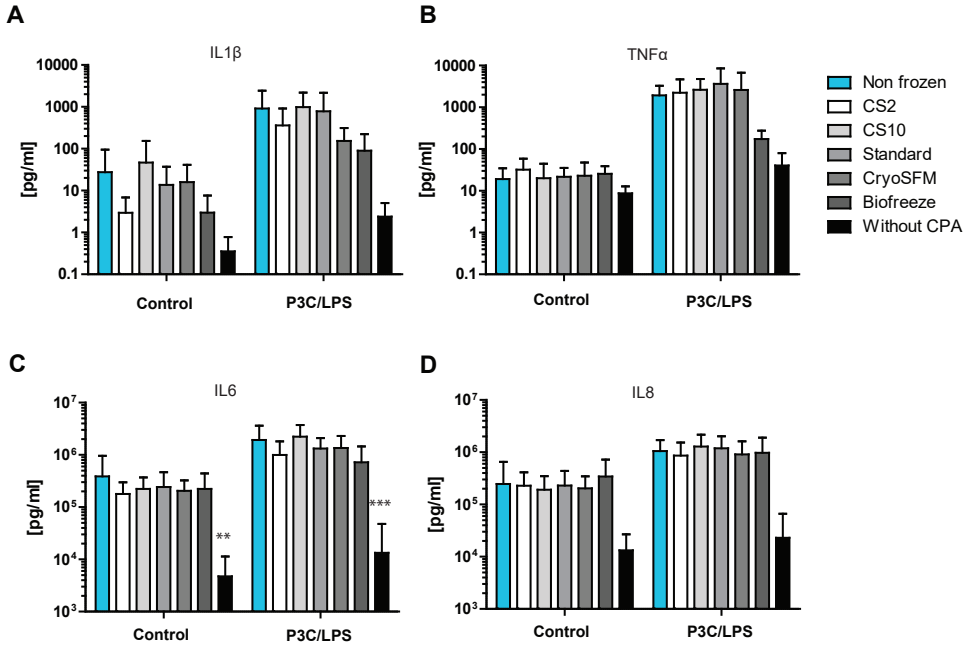

Figure 6. Cryopreserved synovial tissue is responsive to inflammatory stimuli.

The secretion of proinflammatory cytokines IL1β **(A)**, TNFα **(B)**, IL6 **(C)** and IL8 **(D)** was determined before and after 24 hour stimulation with P3C/LPS. Displayed is the mean +/- SD. Statistical analysis was performed by Students t-test, comparing the unstimulated control to the stimulated conditions. ** $p<0.01$; *** $p<0.001$.

Discussion

In this study we validated our slow freezing protocol for human OA synovial tissue by using 4 commercially available and one homemade CPA solution. We determined the viability of the tissue after 24 hours culture at the histological, metabolic, gene expression and protein secretion level. Significantly lower histological scores were detected for the CS2 and Biofreeze medium, indicating these media are suboptimal for maintaining tissue morphology. The other assays performed could not reveal significant differences between conditions. However, in the stress- and disease characteristic gene expression analyses, trends were observed pointing at negative changes in gene expression compared to the non frozen control for the Biofreeze

and CS2 medium as well. With the CS10, Standard and CryoSFM CPA solution only minor differences were observed compared to the non-frozen condition, indicating we have successfully cryopreserved human OA synovial tissue using these CPA solutions. Taking into account that the CS10 and CryoSFM media are produced under GMP conditions, which guarantees standardized and validated quality of CPA solutions, less batch-to-batch variation is expected compared to home-made CPA solutions containing FCS and DMSO.

As described above, the CS2 and Biofreeze medium most often showed a divergence from the non frozen condition. Although the exact composition of the commercially obtained media was unknown to us, the DMSO content of the media was described. The CS10 and standard medium contain 10% DMSO, the CryoSFM medium 7.5%, the Biofreeze medium does not contain DMSO and the CS2 medium only 2%. Without knowing about other differences in composition our results suggest that a low DMSO concentration is not beneficial for the cryopreservation of human OA synovium. The fact that the only difference between the CS2 and CS10 medium is the DMSO concentration strengthens this suggestion.

Our results show a lack of response to LPS/P3C in the conditions cryopreserved without protecting CPA solution. The secretion of IL1β, IL6 and IL8 were not significantly elevated in this condition. However, TNFα was. It might be that freeze-thawing stress in the unprotected tissue causes release of membrane-bound TNFα, which leads to a higher protein concentration, while no new protein is secreted. There are links described between increased cellular stress and higher levels of activated TACE/ADAM17 leading to higher protein concentrations of TNFR1, released together with TNFα.[19]

When comparing the results of the XTT and ATP assay, which both reflect the metabolic activity of the tissue, there was one major difference. The ATP levels in patient 4 are the lowest in all conditions compared to the other patients while in the XTT assay the metabolic activity of patient 4 is comparable or higher compared to the other two patients. It might be that the difference in outcome can be explained by a difference in assay mechanism: the XTT assay is based on the cleavage of the tetrazolium salt XTT, probably at the plasma membrane, to an orange formazan dye by the reducing properties of NADH produced by mitochondria.[20] The ATP assay is based on the conversion of Luciferin to the light emitting Oxyluciferin by Ultra-Glo Luciferase. In this reaction ATP is the missing factor and becomes available after cell lysis. Furthermore, it has been described that cellular stress due to inflammation[21] or hypotonic swelling in lung epithelial cells,[22] leads to active ATP release. Hypotonic swelling might also occur during thawing, upon removal of the

CPA solution. Active release of ATP might be involved in the lowered ATP levels in patient 4.

We evaluated the expression of several known stress genes: The heat shock protein family, of which HSPA1A and HSP27 are members, is known to be induced in response to stress. Although HSPA1A is also described to be downregulated by cryopreservation in bovine blastocysts.[23] Heat shock proteins bind to denatured proteins as chaperones assisting in refolding and prevention of aggregation.[24] In our analysis we could not detect significant differences between conditions analyzing these genes, except for the upregulation of HSPA1A and HSP27 upon cryopreservation in the condition in which the synovial tissue was cryopreserved without CPA. CASP3, BAX, CD95 and MCL1 are apoptosis associated proteins. CASP3, BAX and CD95 are pro-apoptotic proteins while MCL1 is anti-apoptotic. Programmed cell death is a mechanism known to be upregulated when experiencing stressful conditions, including cryopreservation.[10] CASP3, BAX and CD95 were described to be upregulated upon cryopreservation while MCL1 was described to be down-regulated.[25] Similar to the heat shock proteins, no significant differences were identified between the cryopreservation conditions except for the non-protected condition in which an upregulation of all four apoptosis related genes was found, including for the anti-apoptotic gene MCL1. An explanation for this could be that there are differences between cell types in expression of pro- and anti-apoptotic genes resulting in upregulation of pro-apoptotic genes in one cell type and anti-apoptotic genes in different cells, resulting in the observation of upregulation of both genes. All together, analyzing stress gene expression after cryopreservation could only be used to detect major stress responses and was not applicable to identify minor qualitative differences between cryopreservation solutions. In contrast to the stress-related genes, the expression of disease-specific genes TIMP3, CCL18 and MMP9 was not significantly affected by cryopreservation and the only differences were observed between samples of individual patients. TIMP3 is a metallopeptidase inhibitor, suggested to have a protective effect against degradation of articular cartilage *in vivo*.[26] CCL18 is a chemokine belonging to the CC chemokine family. CCL18 is mainly produced by antigen presenting cells, to attract among others T-and B-cells.[27,28] MMP9 is a metallopeptidase involved in OA cartilage degeneration.[29] Our results indicate that these genes can be suitable to detect subtle cryoprotectant or cryopreservation effects other than cryotoxicity at an individual.

In our approach we cultured the tissue for 24 hours after thawing and subsequently performed analysis to determine the viability. This time point was

chosen as it has been suggested by the manufacturer of the BioLife CPA solutions. Furthermore, measuring cellular viability within the first hours after thawing likely results in an overestimation of the viability.[30] Additionally, the 24 hour time point is a common culture time point in functional studies, although some parameters might be best studied at a different time point. For instance, a peak in Caspase 3 expression was described 18 hours post thawing in normal human dermal fibroblasts. However, also at 24 hours post thawing, levels were still elevated compared to the control.[31] We have not included more time points in our analysis due to scarcity of material.

A validated cryopreservation protocol is of high importance in research. In the first place to obtain reproducible results and in the second place cryopreservation offers the possibility to pre-select tissue at certain characteristics to be used in a study. Furthermore, if the same cryopreservation protocol is used it offers the possibility to combine synovial material obtained at multiple locations. An important effect we foresee by improving the availability of human synovial tissue for research is that this could lead to a decrease in the number of experimental animals. The possibility to perform more complex and larger experiments (for example drug compound testing) with human material instead of mice will prevent generation of results that cannot be translated to the human situation. Related to this is the increasing interest in precision medicine,[32-34] followed by an increasing demand for human tissue. Precision medicine includes the adjustment and development of therapies and drugs for specific patient subsets with a similar genetic background, leading to for instance a similar response to treatment. This will improve healthcare by applying the best therapy to each patient, additionally this approach will be more cost effective and prevent possible side effects in patients not benefitting from a therapy. The development of precision medicine and the existing research lines using human synovial tissue of both pharmaceutical companies and universities are dependent on the availability of high quality patient material. By performing mechanistic and intervention studies, the development of new treatment options for OA would likely benefit from the use of cryopreserved synovial tissue.

We showed that a slow freezing cryopreservation protocol using commercially available CPA solutions and a homemade CPA solution can protect human OA synovial tissue from cryodamage. We have validated a protocol to determine the viability of the tissue after cryopreservation, including an extensive analysis of the tissue functionality after thawing. Three CPA solutions were identified that performed equally well. A next step is to perform a reproducibility study, in which other labs will use our cryopreservation protocol to test if our results are

reproducible. Furthermore, this study can be used as a template to investigate the possibility to cryopreserve the even more complex synovial tissue of rheumatoid arthritis patients and isolated primary cells acquired by synovial tissue digestion.

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Supplementary information

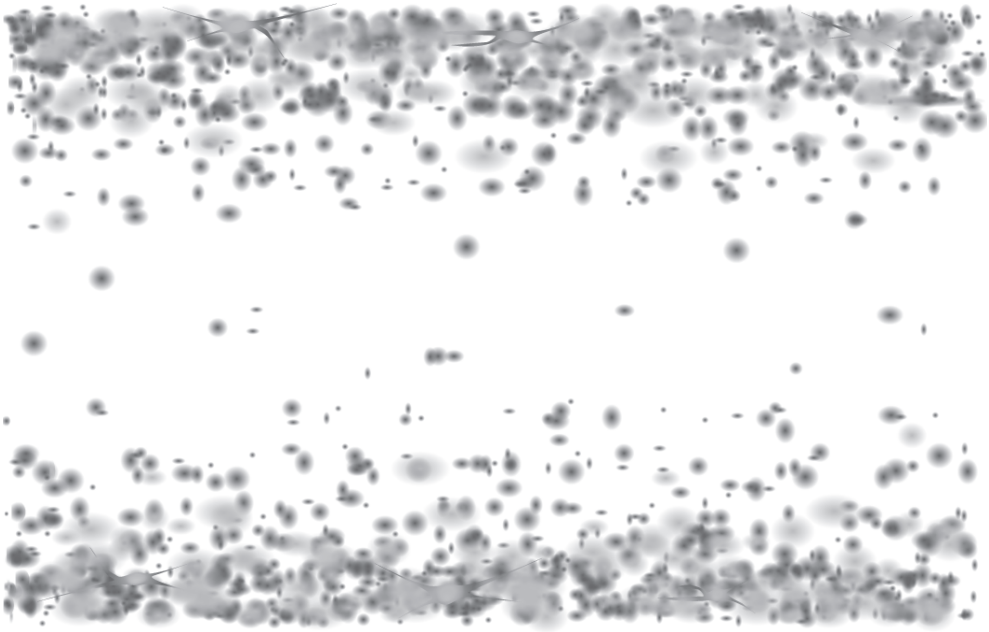
Table S1. List of qPCR primers

Gene symbol	Forward sequence	Reverse sequence
GAPDH	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTGAGC
HSPA1A	CAAGGCCAACAAGATCACC	CTCGATCTCCCCTGCTC
HSP27	CTGGATGTCAACCACTTCG	AGATGTAGCCATGCTCGTC
CASP3	AGCACCTGGTTATTATTCTTGG	TACTGTTTCAGCATGGCAC
BAX	CTTCAGGGTTTCATCCAGGA	TTACTGTCCAGTTCGTCCC
CD95	CTCTTTAAGACTGTTCTTACGTCTG	CTTGGAGTTGATGTCAGTCAC
MCL1	GCTAGTTAAACAAAGAGGCTGG	CTTCTAGGTCCTCTACATGGA
TIMP3	TCTCCTTGTCCCTGCTTCATG	AGGCCCTTGACTACACTCTCATCT
CCL18	GGTGTCATCCTCCTAACCA	GTCGCTGATGTATTTCTGGAC
MMP9	CTTCCAGTACCGAGAGAAAGCCTAT	CAGGACGGGAGCCCTAGTC

Table S2. p-values of increase in cytokine secretion after stimulation

Statistical analysis was performed by Students t-test, comparing the cytokine secretion of control (unstimulated) synovium to P3C/LPS stimulated synovium. Non significant p values are displayed in italics.

Control vs P3C/LPS stimulation	Cytokines			
	IL1β	TNFα	IL6	IL8
Non frozen	0,0431	< 0.0001	0,0041	0,0011
CS2	0,0292	0,0026	0,0021	*0,2380*
CS10	0,0053	0,0001	<0,0001	0,0002
Standard	*0,0506*	0,0120	0,0003	0,0010
CryoSFM	0,0035	0,0256	0,0003	0,0021
Biofreeze	0,0249	< 0,0001	*0,0502*	0,0391
Without CPA	*0,1103*	0,0155	*0,3212*	*0,4334*



Chapter 5

Micromasses of primary synovial cells reassemble into a TNF-alpha-responsive synovial membrane that can be viably cryopreserved.

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In this study, we characterized the synovial membrane model made from micromasses of primary synovial cell suspensions by analyzing the cell composition at several stages of development using immunohistochemical staining and flow cytometry. We observed a gradual decrease in T cells, B cells and endothelial cells, whereas the synovial fibroblasts and synovial macrophages were maintained. Around week 2 of culture the micromasses developed a synovial lining of both macrophages and fibroblasts and showed an acute gene expression response to inflammatory stimuli. Long-term exposure of micromasses to tumor necrosis factor alpha (TNFα) resulted in synovial lining hyperplasia involving both synovial fibroblasts and macrophages. Interestingly, the macrophages in the micromass showed phenotypic plasticity as prolonged TNFα stimulation strongly reduced the occurrence of CD68/CD163-positive M2 macrophages. Micromasses with an established synovial lining can be cryopreserved and after thawing the integrity of the lining remains intact and cellular response to inflammatory stimuli was unchanged. In conclusion, we showed that a large number of micromasses can be made from a single synovial biopsy and that these micromasses spontaneously develop a synovial membrane containing both macrophages and fibroblasts. This synovial membrane model responded to TNFα by enhanced gene expression and hyperplasia. The micromasses are also suitable for cryopreservation and when kept on ice, the micromass dissolves allowing the analysis of individual cells without the requirement of an additional enzymatic digestion. The use of micromasses and the ability to store in biobanks for later use will aid in performing comparative studies using multiple donors simultaneously, and this may have a significant impact on translational arthritis research.

Introduction

Rheumatoid arthritis (RA) and osteoarthritis (OA) are the most common rheumatic diseases. The global incidence of RA is estimated to be 0,5%-1%[1] and an estimated 12.1% of the adult population has clinical symptoms of OA.[2] RA and OA are both viewed as multi-factorial diseases involving multiple risk factors[3,4] but their exact etiology is unknown. Both diseases cannot be cured at present, but disease-modifying drugs (DMARDs) are available for RA and non-steroidal anti-inflammatory drugs (NSAIDs) for OA.

During disease, many processes occur in the synovium. Already in the early phase of RA, hyperplasia of the synovial lining occurs and immune cells infiltrate the sublining.[5, 6] The cells in the synovium can produce several pro-inflammatory cytokines and matrix-degrading enzymes. At the interface of the synovium, cartilage and bone, pannus tissue is formed that contributes to bone destruction. Synovial pathology is also described for OA. Although OA has traditionally been described as a wear-and-tear disease of the articular cartilage, synovitis is observed in up to 50% of the OA patients and might contribute to the disease process.[7] In addition, fibrotic tissue contributes to the synovial thickening in OA,[8] which is also observed as a secondary process in RA.[9] Because hyperplasia, cellular infiltration, joint damage, production of Matrix Metalloproteinases (MMPs), production of cytokines and fibrosis occur in both RA and OA with large patient-to-patient variation, RA and OA have many features in common on histological level.[10] The similarities imply that on local level, the same anti-inflammatory treatment might influence both diseases. One of the cytokines produced by the synovium is Tumor Necrosis Factor alpha (TNFα) and anti-TNF biological drugs are successful in the treatment of RA patients but also a promising strategy in OA.[11,12] However, there is a lack of relevant *in vitro* models to study therapies in these specific disease processes.

Translational arthritis research with human cells is often performed in monolayer culture with *in vitro* expanded synovial fibroblasts. Although monolayer culture with fibroblasts is easy to perform, the absence of extracellular matrix and other cell types result in alterations of cell functioning and a rapid loss of phenotype.[13] One way to maintain the cell composition and matrix interactions of the cells is by using synovial explants instead of cultured monolayer cells. Although synovial explants better represent the original joint environment for the cells, important disease processes including hyperplasia and cartilage damage may have already taken place and can thus not be studied using explants. Moreover, there is considerable variability between biopsies from a single joint, resulting from a

variation in lining thickness,[14] sublining composition and an unequal distribution of synoviocyte types in the synovial membrane.[15,16]

To circumvent these challenges and provide a relevant model for the synovial membrane in which cells can interact with cellular matrix, Kiener and co-workers have developed a 3-dimensional (3D) micromass model based on a mixture of fibroblast-like synoviocytes (FLS) and Matrigel solution. When kept on ice, the Matrigel was liquid and could be mixed with cells to obtain a homogenous solution. Single drops were pipetted in a culture well and became solid at 37°C. Within weeks, the FLS formed a lining layer on the matrigel-medium surface that was reminiscent of a synovial membrane.[17] Reticulin fibers on the surface of the micromasses were arranged in an orientation similar to basement membrane structures in synovial biopsies. The FLS in the micromass lining produced lubricin and showed hyperplasia after 3 weeks stimulation with tumor necrosis factor alpha (TNFα).[18] The lining formation was not observed when using dermal fibroblasts.

We have recently applied the synovial micromass using the complete cell suspension after biopsy digestion for micromass formation[19] without further isolation and purification of the synovial fibroblasts to better mimic their cellular composition and interactions as present in the starting material. In this study, we further characterized the synovial micromasses to study the fate of the different cell types. After lining formation, the micromass mainly consisted out of synovial fibroblasts and macrophages, which also appeared after induction of lining hyperplasia. TNF exposure of the synovial micromasses was used to mimic the inflammatory conditions present in RA and OA. This showed that short-term exposure to TNFα already leads to proinflammatory gene expression and long-term exposure to hyperplasia. A valuable property is that the micromasses could be cryopreserved without loss of structural integrity and biological response. In conclusion, these results provide a detailed analysis of the synovial micromasses and show the suitability as a synovial membrane model for translational research in RA and OA.

Materials and methods

Patient material

Synovial biopsies from 4 RA patients and 2 OA patients were obtained from the Orthopedics department (Radboud university medical center, Nijmegen, the Netherlands) and the Sint Maartenskliniek, Nijmegen, the Netherlands during

joint replacement surgery. All patients signed an informed consent and the research protocols were approved by the local committee on research involving human subjects (CMO region Arnhem-Nijmegen, the Netherlands) under NL-number 54055.091.15. The presence of a synovial lining was determined on 7 µm cryosections stained with hematoxylin and eosin staining (HE) to confirm the synovial origin of the tissue (not shown).

Micromass production

Micromasses were produced as described previously.[19] In short, the biopsies were digested using Liberase (Roche, Basel, Switzerland) for 1h at 37°C and the cells were filtered through a 70 µm cell strainer (Corning, NY, USA). Red blood cells (RBCs) were lysed for 2 min at RT in 4 ml RBC lysis buffer (155 nM NH_4Cl, 12 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). The cells were mixed on ice with liquid Matrigel and 25 µl droplets containing ~$5x10^5$ cells were pipetted on a poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich, Zwijndrecht, The Netherlands) coated 24-wells plate (Greiner Bio-one, Alphen a/d Rijn, the Netherlands). After 30 min gelation at 37°C, 500 µl RPMI medium supplemented with 10% fetal calf serum (FCS), 1 mM pyruvate and 1% penicillin/streptomycin (P/S) was added. The micromasses were cultured for the time and conditions as indicated in the separate Figures. In general, medium was replaced twice weekly and micromasses were cultured at 37°C and 5% CO_2.

Flow cytometry

Micromasses were kept on ice for 2h and the cells were washed to remove the liquid Matrigel. For 11-Fibrau staining, cells were incubated with 11-Fibrau (1:50 for 60 minutes) (clone D7-fib, Imgen, distributed by ITK Diagnostics, Uithoorn, The Netherlands) and subsequently with donkey-anti-mouse conjugated to Alexa Fluor 568 (1:100 for 30 minutes) (A-10037, Thermo Scientific). CD90 was stained using mouse anti-human CD90-APC (1:20 for 30 minutes) (17-0909 eBioscience, San Diego, CA, USA). CD68 was stained using mouse anti-human CD68-PE (1:20 for 60 minutes) (12-0689, eBioscience); CD14 was stained using mouse anti-human CD14-APC (1:20 for 60 minutes) (301808 Biolegend, San Diego, CA, USA). The samples were measured on the CyAn ADP analyser (Beckman Coulter, Woerden, The Netherlands) using the 488 nm laser at the FL1, FL7 and FL8 channels for GFP, Alexa Fluor 568/PE and APC respectively.

Immunohistochemistry

Micromasses were fixated in 2% paraformaldehyde in phosphate-buffered saline

(PBS)/1mM CaCl2 for 2h, dehydrated and embedded in paraffin. 7 µm sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE) or with primary antibodies for 60 minutes: 11-Fibrau (1:100), mouse anti-human CD68 (1:100 for 60 minutes, M0814, DAKO, Heverlee, Belgium), mouse anti-human CD20 (1:100 for 60 minutes, 120M-84, Cellmarque, Rocklin, CA, USA), mouse anti-human CD3 (1:50 for 60 minutes, M0814, DAKO, Heverlee, Belgium), rabbit anti-human CD31 (1:50 for 60 minutes, GTX48364, Genetex, Hsinchu City, Taiwan), mouse anti-human CD163 (1:25, BAM16072, R&D systems, Oxford, UK). Isotype control antibodies were Mouse IgG1κ (X0931, DAKO), mouse IgGακ (X0943, DAKO) and Rabbit IgG (X0936, DAKO). Endogenous Peroxidase activity was blocked with 3% H2O2 (Merck Millipore, Amsterdam, The Netherlands) in methanol. Subsequently, the murine primary antibodies were stained with HRP-conjugated rabbit-anti-mouse IgA/G/M (1:200 for 60 minutes, P0260, DAKO) and the rabbit primary antibodies were stained with HRP-labeled goat anti-rabbit-Ig(all) (1:200, P0448, DAKO). Peroxidase was developed with diaminobenzidine and counterstained with hematoxylin for 60 seconds. For CD68-staining of synovial cryosections, primary antibody mouse anti-human CD68 (1:100, M0718, DAKO) was used and for CD3 staining of synovial cryosections rat anti-human (1:50, MCA1477, Bio-Rad, Puchheim, Germany).

Image analysis

Microscopic images were taken using the Leica DMR light microscope after randomization of the samples. For hyperplasia analysis, 3 sections were analyzed per micromass and per section 3 pictures were taken from the micromass surface after 11-Fibrau staining. The lining hyperplasia was scored based on an arbitrary scale ranging from 0 (the surface with the fewest cells) to 3 (most cellular surface). For the counting of the large CD68 and CD163-positive cells, 2 pictures from 3 sections per micromass were analyzed using the Leica Application Suite (LAS) software. The total number of large cells was counted and divided by the surface area of the micromass in the analyzed image.

Micromass cryopreservation and viability assay

Cryopreservation of micromasses was performed using the protocol previously described for synovial biopsies.[20] In short, micromasses in which the lining had formed were submerged in 1 ml Cryostor CS10 medium (Sigma-Aldrich) and incubated on ice for 10 min. Subsequently, the micromasses were transferred to -80°C in a Mr. Frosty isopropanol container (Thermo Scientific) pre-incubated at 4°C. After overnight cooling, the micromasses were transferred to liquid nitrogen.

After 7 days, the micromasses were thawed and washed 3 times in 1 ml PBS. After 24h acclimatization in 200 µl supplemented RPMI medium in a 96-wells plate, the viability was assessed using the cell titer Glo kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations. The non-frozen control micromass viability was assessed after 24h culture in a 96-wells plate.

Gene expression analysis

The micromasses were dissolved in 500 µl Tri reagent (Sigma-Aldrich) and total RNA was isolated according to the manufacturer's protocol. qPCR was performed as previously described [21]. The following primer sequences were used: GAPDH forward primer: ATCTTCTTTTGCGTCGCCAG, GAPDH reverse primer: TTCCCCATGGTGTCTGAGC. IL-1β forward primer: TGGGTAATTTTTGGGATCTACACTCT, IL-1β reverse primer: AATCTGTACCTGTCCTGCGTGTT, IL-6 forward primer: AGCCCACCGGGAACGA, IL-6 reverse primer: GGACCGAAGGCGCTTGT, TNFα forward primer: TCTTCTCGAACCCCGAGTGA, TNFα reverse primer: CCTCTGATGGCACCACCAG. Values are depicted as threshold cycle, corrected for GAPDH expression.

Statistical analysis

Statistical analysis was performed by Student's t-test, 1-way ANOVA or 2-way ANOVA as indicated in the figure legends using Graphpad Prism software v5.03. p-values below 0.05 were regarded as significant.

Results

Analysis of cell synovial cell suspensions

The composition of the cell suspension after digestion of a synovial biopsy was determined using cell surface markers. After 24h culture we observed cells positive for synovial fibroblast marker 11-Fibrau (70.0%), fibroblast/endothelial cell marker CD90 (68.9%), monocyte/macrophage marker CD68 (24.0%) and monocyte marker CD14 ($CD14^{low}$ 22,6% and $CD14^{high}$ 6,48%) **(Fig. 1A)**. This cell composition was comparable in repeat experiments. No T cells (CD3), B cells (CD20) or dendritic cells (CD11c) were observed in this cell suspension (data not shown). Cells that were stained positive with CD90 also stained positive for 11-Fibrau. Almost all CD68-positive cells also stained positive for CD14. The CD68-positive cells were $CD14^{low}$.
After 72h culture, the cell composition was re-analyzed to determine the culture effects on the cell suspension. We observed a minor decrease in

11-Fibrau and CD90-positive cells (to 65.5% and 67.8% respectively) and a slight increase in CD68-positive cells (to 27.3%) **(Fig. 1B)**. The CD14^{high} fraction dropped to 3,19% and the CD14^{low} to 13.1% after 72h culture. The distinct CD68-positive and CD14^{low} cells lost most CD14 expression after 72 hours

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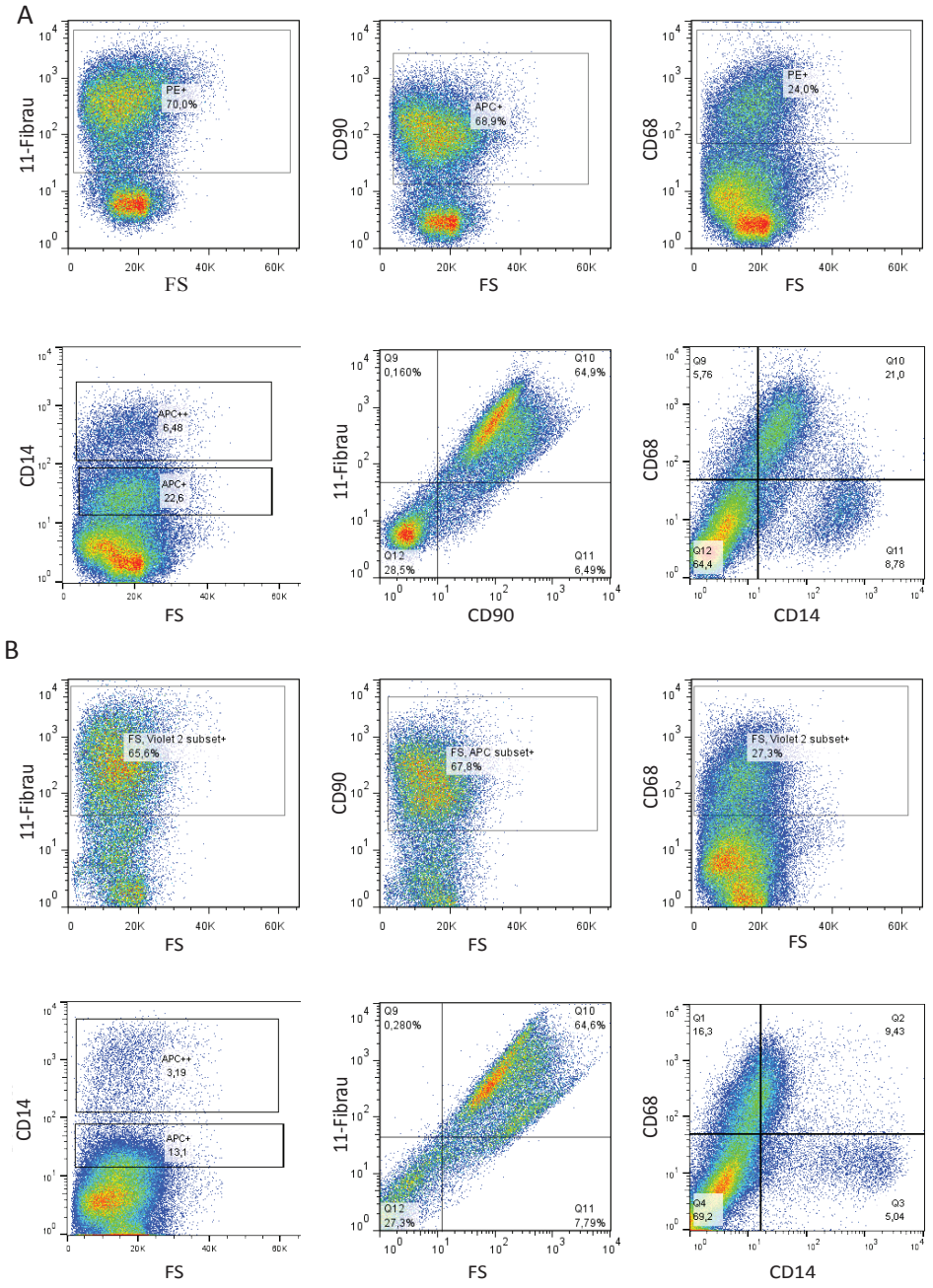


Figure 1: Flow cytometry analysis of cell suspension after biopsy digestion.
An RA synovial biopsy was digested using Liberase. Cells were cultured for 24 hours **(A)** or 72 hours **(B)**. The cell suspensions were subsequently stained with markers for synovial fibroblasts (11-Fibrau), endothelial cells (CD90), macrophages (CD68) and monocytes (CD14). FS: forward scatter.

Kinetics of micromass lining formation and cell type composition

The kinetics of lining formation in the synovial micromasses was assessed at different time points. 1 day after biopsy digestion and micromass production, the cells had not regained their original morphology and all cells were round-shaped **(Fig. 2)**. The first time point at which a distinct lining was visible, was after 14 days. In multiple experiments we observed lining formation between day 7 and day 14. In contrast, lining formation in micromasses from cultured FLS was typically completed between day 5 and day 7 (data not shown). The synovial lining remained visible until the end of the experiment at day 35.

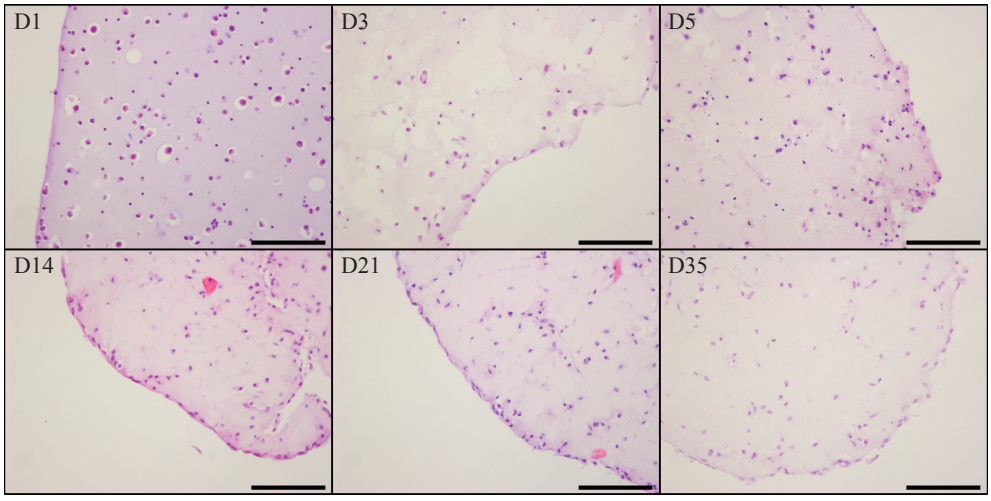


Figure 2: Kinetics of micromass formation.
Micromasses were generated from an RA synovial biopsy. At different time points (day 1, 3, 5, 14, 21 and 35) micromasses were fixed and embedded in paraffin. 7 µm sections were stained with HE. Pictures were taken at 200x magnification and black scale bars represent 100 µm.

We analyzed the composition of the synovial micromasses at several time points during development both by flow cytometry and histology. For flow cytometry analysis of mature micromasses after lining formation, the micromasses were

incubated on ice for 2h. The Matrigel re-liquidized and was washed away prior to cell staining. The composition of the micromass was comparable to the cultured cell suspension described in Figure 1 **(Fig. 3)**. We observed adequate amounts of cells positive for 11-Fibrau, CD68 and CD90. The CD68-positive cells showed only low expression of CD14, reminiscent of the culture effects observed with culture of the cell suspension. These results show that the mixed cell population is maintained during micromass culture.

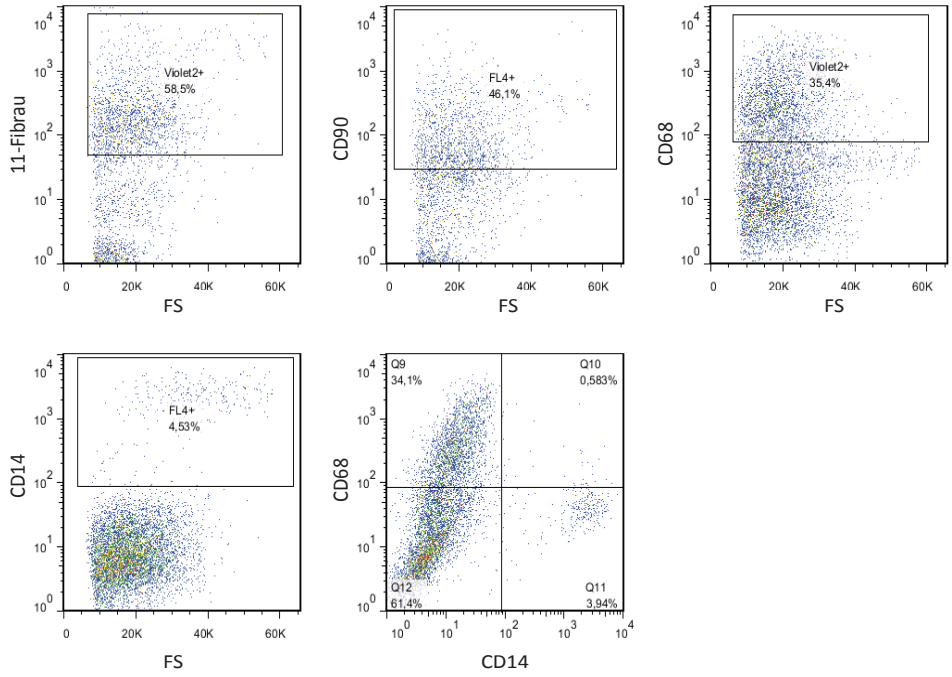


Figure 3: Flow cytometry analysis of micromass cell composition after lining formation. Micromasses from RA biopsies were cultured for 7 days until lining formation and subsequently incubated on ice until the Matrigel became liquid. The cells were stained with 11-Fibrau, CD90, CD68 and CD14. Similar profiles were obtained in multiple experiments. FS: forward scatter.

In the synovial biopsy, cells that stained positive for macrophage-marker CD68 were primarily localized in the lining of the synovium **(Fig. 4)**. Immediately after formation of the micromasses, CD68-positive cells appeared randomly distributed over the complete micromass. During the first week of micromass culture, a gradual increase in the relative amount of CD68-positive cells was observed and after 7 days most CD68-positive cells appeared as large cells. The analyzed biopsy also contained foci of B cells (CD20-positive) and T cells (CD3-positive) **(Fig. 4)**.

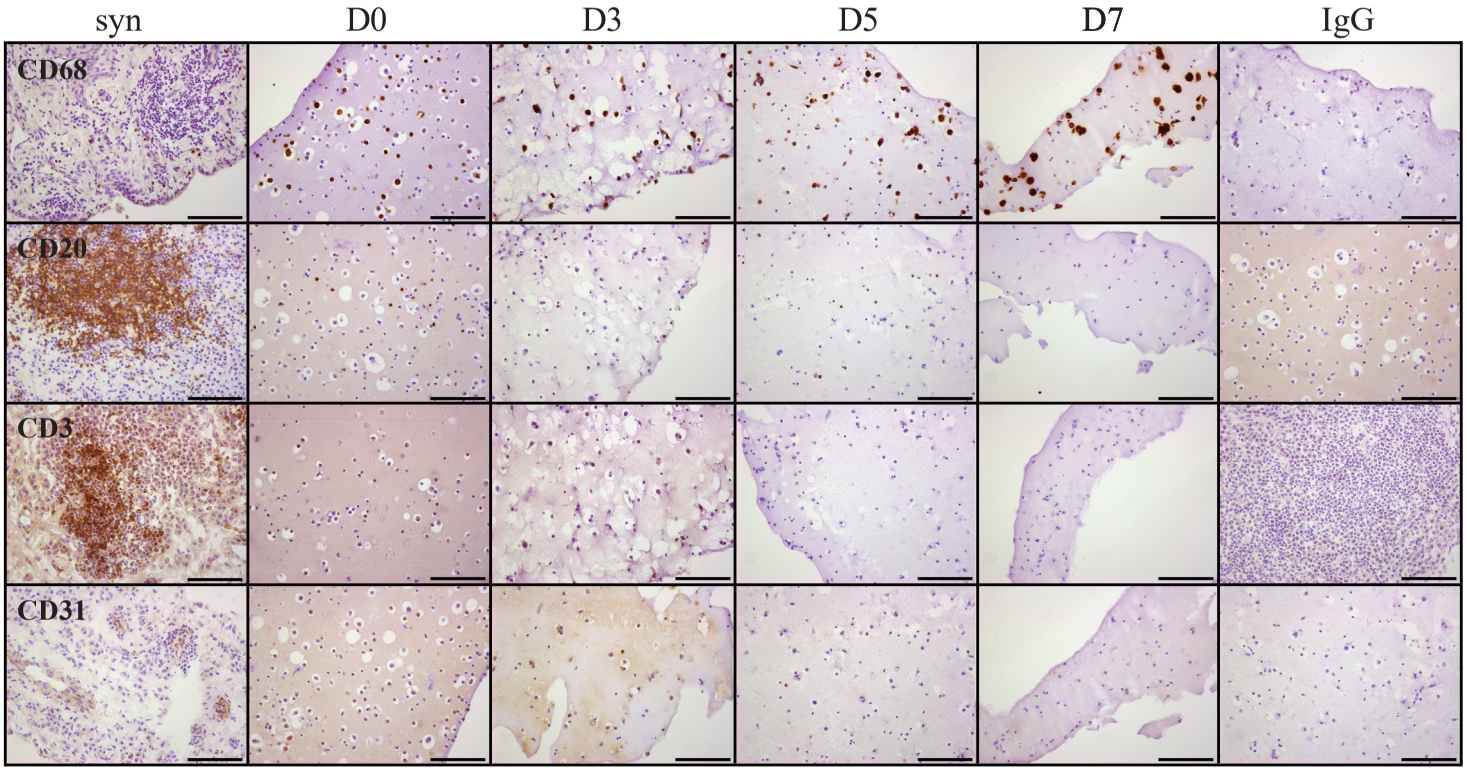


Figure 4: Cell type composition during micromass formation.

Synovial biopsies were divided into several pieces. One piece was snap frozen in Tissue-Tek O.C.T. (syn) and cryosections were stained for synovial macrophage marker CD68, B cell marker CD20, T cell marker CD3 and endothelial cell marker CD31. The remaining pieces were digested and used for micromass formation. At day 0, day 3, day 5 and day 7 micromasses were fixed and embedded in paraffin. 7 µm sections were stained for cell markers. Pictures were taken at 200x magnification and black scale bars represent 100 µm. IgG: isotype IgG negative control.

At lower frequency compared to CD68-positive cells, these cells were incorporated in the micromass. We observed a gradual decrease in the number of B cells and T cells and after 7 days none could be observed. Similar results were obtained with endothelial marker CD31, which stained the blood vessels in the biopsy cryosections **(Fig. 4)**.

Short-term exposure of synovial micromasses to inflammatory stimuli leads to inflammatory gene expression.

We established the inflammatory response of mature micromasses (i.e. micromasses with a completed lining) after stimulation with recombinant human IL-1β, TNFα and lipopolysaccharide (LPS). We observed a significant increase in expression of IL-1β and IL-6 with all three stimuli **(Fig. 5A, B)**. The upregulation of TNFα mRNA was only significant after LPS stimulation **(Fig. 5C)**. These results show that the micromasses are sensitive to inflammatory triggers.

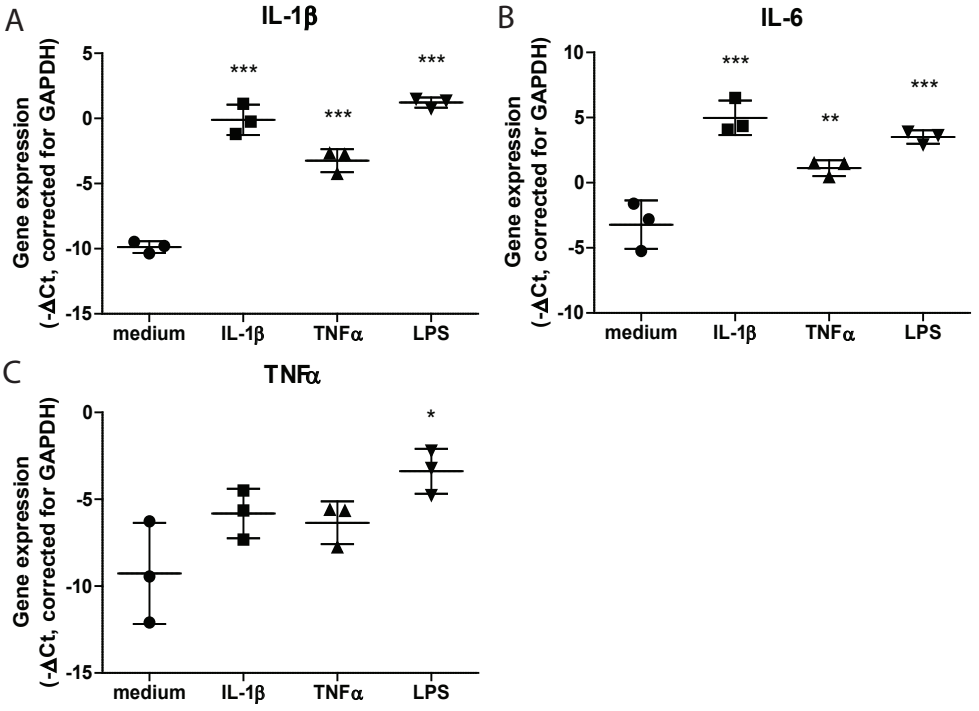


Figure 5: Gene expression after short-term micromass exposure to inflammatory stimuli. Micromasses were generated from an RA knee biopsy and cultured until lining formation was complete. Subsequently, the micromasses were stimulated for 6h with regular culture medium, 10 ng/ml IL-1β, 10 ng/ml TNFα or 100 ng/ml LPS. The gene expression of pro-inflammatory cytokines IL-1β

(A), IL-6 **(B)** and TNFα **(C)** were measured by qPCR. Gene expression values were corrected for GAPDH expression and are depicted as mean +/- SD. Symbols represent individual micromasses. Expression levels were compared to medium condition by 1-way ANOVA and Dunnett's multiple comparison test. * =P<0.05, ** =P<0.01, *** =P<0.001.

Long-term TNFα exposure of synovial micromasses leads to hyperplasia

An important feature of arthritis is synovial hyperplasia, which is hard to study in biopsies. The hyperplasia and cell composition of the lining were assessed after 3 weeks of repeated stimulation with TNFα. Without TNFα stimulation, the lining thickness was on average 1 cell layer **(Fig. 6A)**. After long-term stimulation with TNFα, the lining thickness increased **(Fig. 6B)**. The cells in the lining were positive for 11-Fibrau **(Fig. 6C)** and CD68 **(Fig. 6D)**, indicating that after hyperplasia in the synovial micromass model, the lining contains both FLS and macrophage-like cells. The hyperplasia was quantified using an arbitrary scoring system (0-3) based on the average lining cellularity of 9 images per micromass. The cellularity was significantly increased after TNFα stimulation **(Fig. 6E)**.

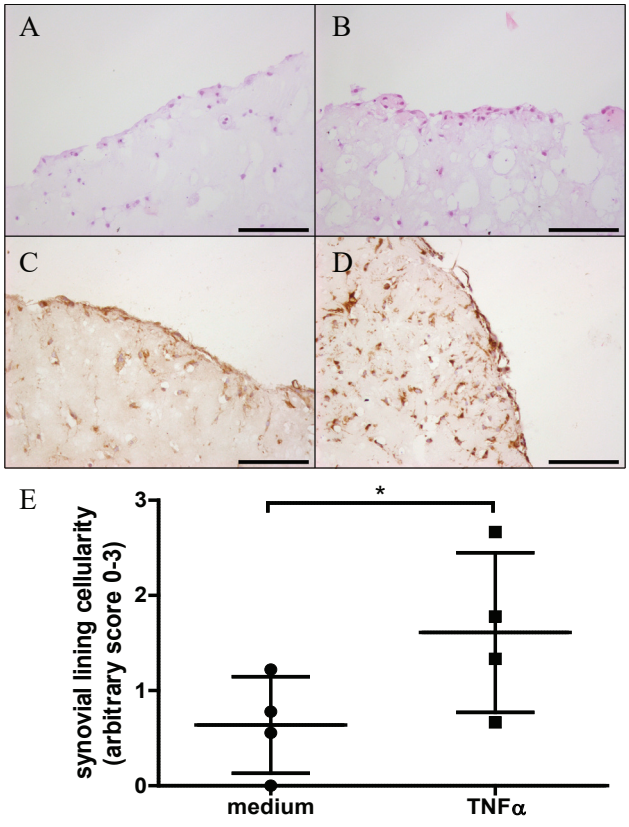
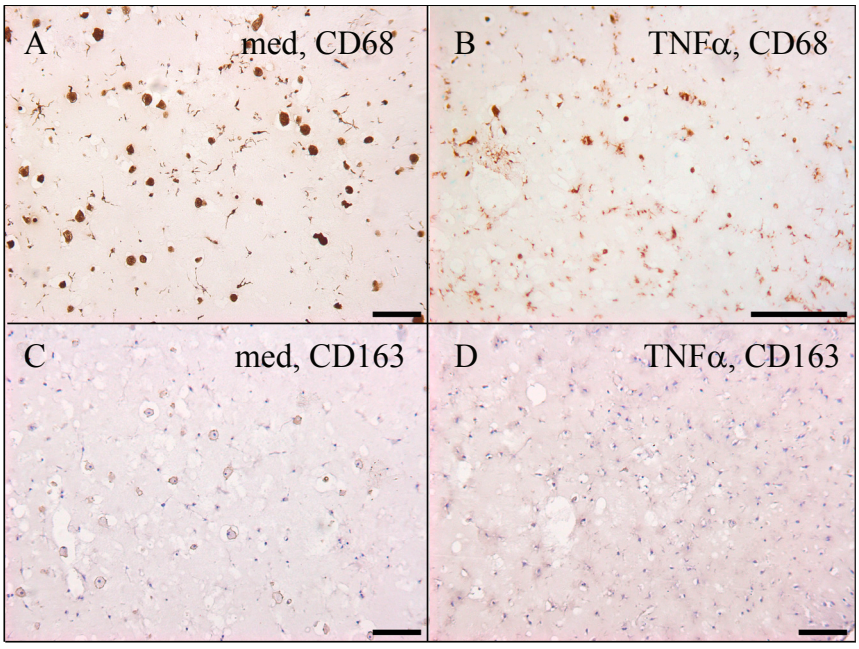
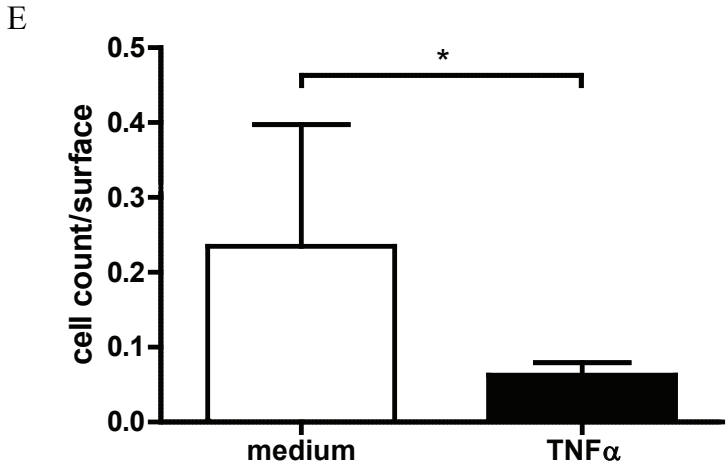


Figure 6: Micromass lining hyperplasia. Micromasses were generated from OA synovial tissue. At day 7, micromasses received 10 ng/ml TNFα for 3 weeks, which was replaced 2 times per week. Control micromasses received culture medium without TNFα. **(A)** HE-staining of micromasses stimulated without TNFα. **(B)** HE-staining of micromasses stimulated with TNFα. **(C)** 11-Fibrau staining of micromasses after hyperplasia. **(D)** CD68-staining of micromasses after hyperplasia. **(E)** Quantification of lining cellularity after hyperplasia. Symbols indicate separate micromasses and the average score of 3 images per 3 slides of every micromass (one dot represents the average of 9 images from one micromass). Statistical analysis was performed by Student's t-test. * =P<0.05.

In addition to effects on lining hyperplasia, we observed striking differences in the cells residing in the micromass matrix. When the micromasses were not treated with TNFα, some cells positive for CD68 showed a hypertrophic phenotype (larger) **(Fig. 7A)**. These cells were not observed after stimulation with TNFα **(Fig. 7B)**. At the location of the cell membrane, we observed positive staining for CD163, a membrane marker associated with type 2 macrophages **(Fig. 7C,D)**. The appearance of these cells could be abolished by TNFα stimulation **(Fig. 7E)**.

Figure 7: Macrophage phenotype after TNFα stimulation. Micromasses were stimulated for 3 weeks with medium (A,C) or TNFα (B,D) and stained with CD68 **(A,B)** or CD163 **(C,D)**. **(E)** Quantification of large CD68-positive cells. Values were corrected for the total surface area of the images analyzed. Values are depicted as mean +/- SD Statistical analysis was performed by Student's t-test. n=4/group * =P<0.05.





Cryopreservation of synovial micromasses

An important challenge in the application of primary patient material is the sparse availability of the biopsies, which greatly hampers the planning and synchronization of experiments with biopsies from multiple patients. We therefore determined the physiological response of cryopreserved and non-frozen synovial micromasses. After cryopreservation, the metabolic activity was measured, but, insufficient samples were analyzed to enable statistical analysis and draw solid conclusions **(Fig. 8A)**. The synovial lining integrity on histological sections were similar in cryopreserved

micromasses compared to non-frozen micromasses **(Fig. 8B)**. To assess the inflammatory response after cryopreservation, we compared the gene expression data from Figure 5 to micromasses from the same patient that received the same pro-inflammatory stimuli, but were cryopreserved between lining formation and 6h stimulation. Similar to non-frozen micromasses, the cryopreserved micromasses were responsive to all three tested stimuli **(Fig. 8C)**. These results show that micromasses can still respond to pro-inflammatory triggers after cryopreservation similar to non-frozen controls.

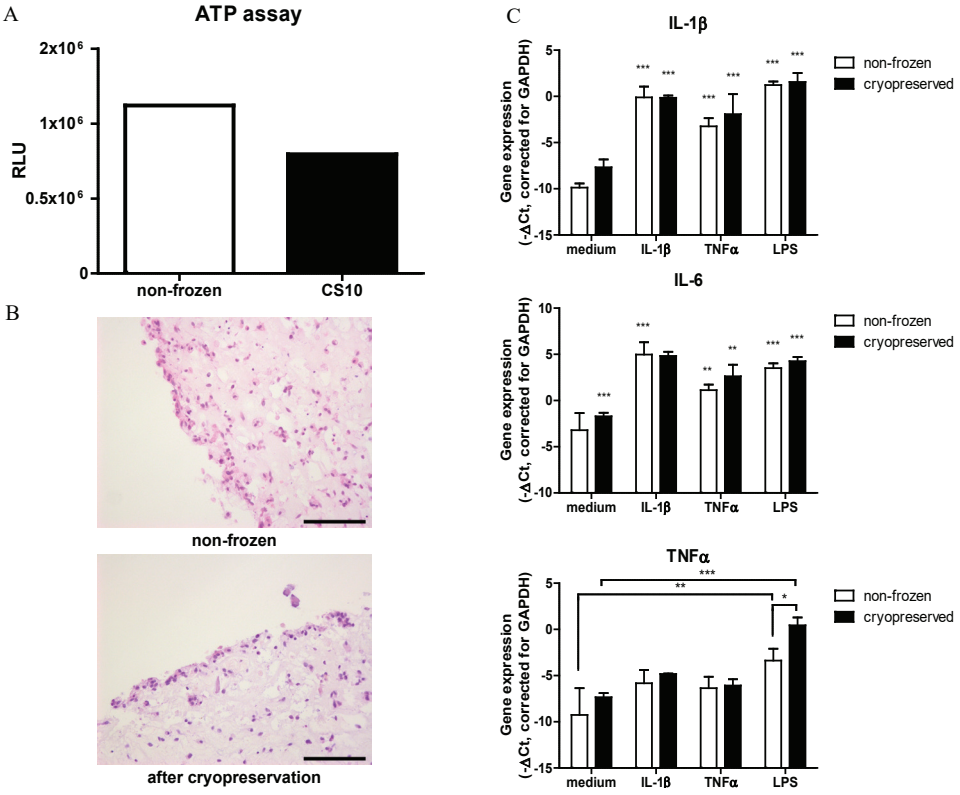


Figure 8: cryopreservation of synovial micromasses. (A) ATP viability assay before and after cryopreservation with CS10. Values are the average of duplo measurement and depicted as relative light units (RLU). **(B)** HE-staining of paraffin sections of non-frozen and cryopreserved micromasses. **(C)** Gene expression of cytokines IL-1β, IL-6 and TNFα after 6h stimulation with recombinant human IL-1β, TNFα or LPS. Gene expression values were corrected for GAPDH expression and are depicted as mean +/- SD. Comparisons within groups were performed by 2-way ANOVA and comparisons between groups by 1-way ANOVA, n=4/group. Significanties indicate a comparison to the appropriate medium-stimulated control. * =P<0.05, ** =P<0.01, *** =P<0.001.

Discussion

In this study, we further characterized the 3D synovial micromass model produced from primary human biopsies for application in both RA and OA research. The model was first explored by Kiener and co-workers, based on primary synovial fibroblasts (FLS) culture. The experiments were focused on cell-cell and cell-matrix interactions during the formation of the lining and the involvement of Cadherin-11.[17,22] We have constructed micromasses from digested biopsies containing the complete cell composition of the synovium. We observed that during culture of the micromass, 11-Fibrau-positive fibroblasts and CD68-positive macrophages migrate towards the lining and can survive for at least 35 days. These 2 cell types correspond with the predominant cell types observed in the intimal layer of the synovium, the type B synoviocyte (FLS) and type A synoviocyte (macrophage-like synoviocyte) respectively.[23] In addition, the CD3-positve T cells, CD20 positive B cells and CD31 or CD90-positive endothelial cells mainly derived from the subintimal synovium are present in the initial Matrigel mixture, but disappear during the first 7 days of culture.

The behavior of the different cell types may in part be explained by the composition of the Matrigel extracellular matrix. Matrigel is an extract of Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, cultured as a tumor in mice. The extracellular matrix produced in high quantities by these cells contains proteins associated with the basal membrane, including Collagen type IV, laminin and perlecan.[24] Cells cultured on or in Matrigel showed increased differentiation and migration. This behavior might be caused by the presence of different growth factors in the Matrigel, including transforming growth factor beta (TGFβ), epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF).[25] The loss of T and B cells is likely to be related to the absence of survival factors like IL-2,[26] which should be added during culture to study the T cells and B cells in mature micromasses. Moreover, the presence of T cells and B cells in the starting biopsy should first be determined, because T cells and B cells were only observed in a portion of the biopsies.

The predominant survival of FLS and macrophage-like cells was observed both by immunohistochemistry and flow cytometry. Several results imply changes in the macrophage population. During culture, the amount of $CD68^+$ cells expressing CD14 decreased at day 3 compared to day 1. The same decrease was observed in macrophages at day 7 after formation of the micromasses. This can be the result of macrophage differentiation, which is associated with a decrease in CD14

expression.[27] During the 3-week micromass culture, we observed large $CD68^+$ and $CD163^+$ cells. These cells represented an M2-like anti-inflammatory macrophage phenotype.[28,29] The appearance of these cells was abolished by stimulation with TNFα, which is known to polarize the macrophages to a pro-inflammatory M1-like phenotype.[30] Although additional markers are required for more accurate and reliable identification of specific cell types and cell subsets, the results clearly indicate a plasticity in the macrophage-like cell population in the synovial micromass.

Both the short-term effects of TNFα and the long-term effects on micromasses were evaluated. In addition to the well-known role of TNFα in RA, the TNFα produced by the osteoarthritic synovium is strongly associated with systemic low-grade inflammation and production of matrix-degrading enzymes by multiple cells in the joint.[31,32] The observation that systemic TNFα levels are increased prior to the most pronounced cartilage damage supports the hypothesis that inflammation in OA is a cause rather than a consequence. We observed that the pro-inflammatory effects can be studied in the micromasses after short stimulation with pro-inflammatory triggers. In addition, synovial hyperplasia is observed in the synovium of both RA and OA patients.[33] We could re-create this pathogenic process by stimulating the micromasses for 3 weeks with TNFα, which is a major advantage compared to studies using synovial biopsies, in which hyperplasia has already occurred.

Interestingly, we found that when kept on ice, the Matrigel liquidized and could be washed away from the cells. The resulting cell suspension could be analyzed by flow cytometry without the requirement of enzymatic digestion. This further enhances the applicability of the micromass model. When performing flow cytometry, we noticed a cell population with low forward and side scatter which was not included in the live gate in our experiments. These cells were not stained by any of the tested membrane markers and could be removed by Zapoglobin treatment, indicating that these cells were red blood cells not completely lysed during micromass production. However, the Zapoglobin treatment also affected the flow cytometry stainings and we recommend for future studies to exclude these cells from the live gate.

The observation that cryopreservation of the synovial micromasses does not influence the histological integrity or the inflammatory response is important for future applications. The availability of biological material for the production of synovial micromasses is dependent on the planning of surgery and therefore sporadic and highly variable. This provides particular challenges for experiments in

which fresh material of multiple patient donors needs to be included simultaneously. By cryopreservation of the micromasses, acquired biopsies can be processed immediately and surplus micromasses can remain in storage for future experiments. In addition, cryopreservation might facilitate the possibility to ship micromasses to different facilities and support multi-center studies.

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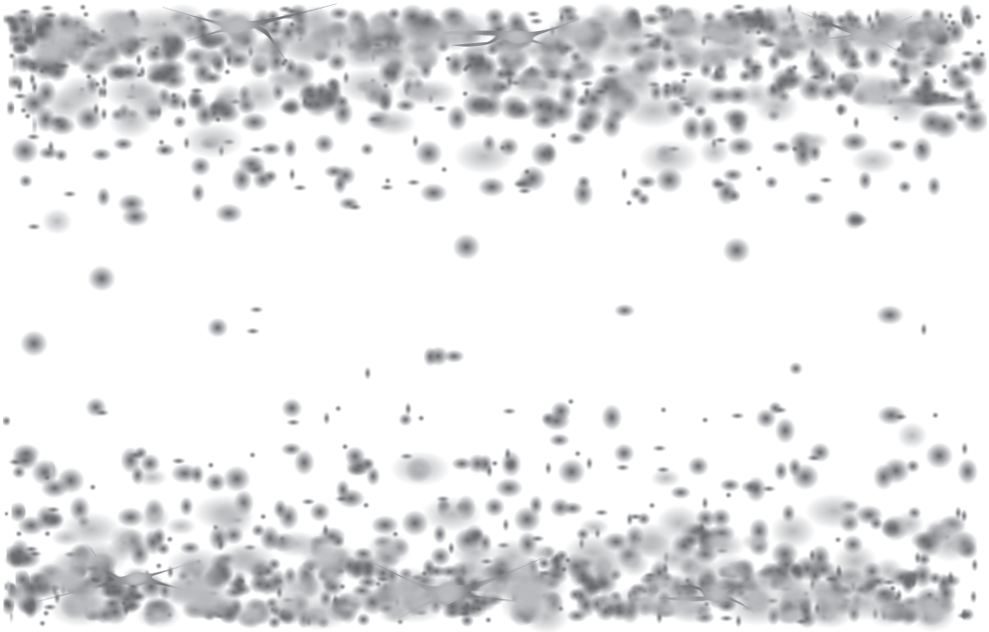

Chapter 6

Disease-regulated gene therapy with anti-inflammatory interleukin-10 under control of the CXCL10 promoter for the treatment of Rheumatoid Arthritis.

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Objective:
Disease-inducible promoters for the treatment of rheumatoid arthritis (RA) have the potential to provide regulated expression of therapeutic proteins in arthritic joints. In this study, we set out to identify promoters of human genes that are upregulated during RA and are suitable to drive the expression of relevant amounts of anti-inflammatory Interleukin-10 (IL-10).

Methods:
Microarrays on RA joint tissues was compared to healthy controls. The *CXCL10* promoter was obtained from human cDNA and cloned into a lentiviral vector containing firefly luciferase. The promoter inducibility was determined in primary synovial cells and in THP1 cells. The luciferase gene was replaced with IL-10 to determine the therapeutic properties of the CXCL10p-IL10 lentiviral vector.

Results:
Microarray analysis yielded a list of 22 genes upregulated during RA. Of these genes, *CXCL10* showed the highest induction in LPS-stimulated synovial cells. The promoter activation was strongest at 8-12h after stimulation with the pro-inflammatory cytokine TNFα and was re-inducible after 96h. The *CXCL10* promoter showed a significant response to RA patient serum, compared to serum from healthy individuals. Primary synovial cells transduced with CXCL10p-IL10 showed a great increase in IL-10 production after stimulation, which reduced the release of pro-inflammatory cytokines TNFα and IL-1β.

Conclusion:
The selected proximal promoter of the *CXCL10* gene responds to inflammatory mediators present in serum of RA patients and transduction by the lentiviral *CXCL10*p-IL10 vector reduces inflammatory cytokine production by primary synovial cells from RA patients. *CXCL10* promoter-regulated IL-10 overexpression can thus provide disease-inducible local gene therapy suitable for RA.

Introduction

Gene therapy for rheumatoid arthritis (RA) enables transduced joint cells to express biological drugs. Because the therapy can be applied locally and requires fewer injections, the side effects of continuous systemic drug administration can be reduced. In addition, off-target effects can be even further reduced by using a disease-responsive promoter that is only active during disease flares and silent during remission.[1]

Derivatives of currently applied biological drugs have been tested as a potential gene therapy in multiple clinical studies after successful application in animal models of RA, including the Interleukin-1 receptor antagonist (IL1-RA) and the soluble extracellular domain of the TNF receptor fused to an IgG tail.[2,3] No severe adverse side effects were reported that could be contributed to the gene therapies.[4] However, only minor clinical improvements were observed. These results solicit for the use of a more potent anti-inflammatory transgene, like Interleukin-10 (IL-10). IL-10 has pleiotropic effects and can inhibit the development of Th1 cells and the production of multiple pro-inflammatory cytokines as comprehensively reviewed by Bijjiga and Martino.[5]

Clinical studies with recombinant IL-10 are impeded by the short half-life of IL-10 *in vivo*.[6] In addition, Crohn's disease patients systemically treated with high doses of recombinant IL-10 showed a significant reduction in hemoglobin levels.[7] These issues might be circumvented in RA by using local gene therapy. Recent studies have successfully attempted to provide disease-inducible expression of IL-10 in murine experimental arthritis.[8,9] The promoters that were tested for therapeutic application with IL-10 in these studies were the IL1E-IL6P, a combination of the *IL-1β* enhancer element and the *IL-6* promoter, the *Saa3* promoter and the *Mmp13* promoter.

The translation of the use of inducible promoters from experimental arthritis to the human situation is hampered by both differences in the disease process and the subsequent activation of the associated genes, as well as the differences in the sequences of homologous promoters. For instance, the *Saa3* promoter showed the highest inducibility in previous studies,[10] but is only present as a pseudogene in humans.

In this study, we show the upregulation of the human *CXCL10* promoter in joint tissue of RA patients and the inducibility of the isolated *CXCL10* promoter in THP-1 monocytes and in primary synovial cells. When the *CXCL10* promoter was used to drive the transcription of the *IL-10* transgene in a lentiviral vector, IL-10 production was significantly increased in cells stimulated with pro-inflammatory cytokines. The

increased production of IL-10 resulted in a decreased release of pro-inflammatory cytokines by synovial cells, indicating the potential of the CXCL10p-IL10 construct to provide a disease-regulated production of anti-inflammatory IL-10.

Materials and methods

Patient material

Synovial samples for microarrays were obtained during surgery or by fine-needle arthroscopy and stored in liquid nitrogen until further processing. In total, an additional 8 synovial biopsies for digestion were obtained during surgery from the Department of Orthopedics (Radboud University Medical Center, Nijmegen, The Netherlands) or the Sint Maartenskliniek, Nijmegen, The Netherlands. Synovial biopsies were transported in DMEM culture medium, supplemented with 1% penicillin/streptomycin at 4°C. First, the pieces were confirmed to contain a synovial lining by histological analysis of cryosections. Examples of the sample cryosections are shown in Figure S1.

Subsequently, the pieces were digested with 50 µg/ml Liberase TM (Roche, Basel, Switzerland) for 1h at 37°C in plain RPMI culture medium. The digestion was quenched by adding 10% fetal calf serum (FCS). Subsequently, the synovial cells were passed through a 70 µm cell strainer (Corning, NY, USA) and centrifuged for 5 min at 1500 rpm. Red blood cells were lyzed using RBC lysis buffer (155 nM NH_4Cl, 12 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 2 min at RT.

Sera from RA patients were obtained from early RA at the Department of Rheumatic Diseases, Radboud University Medical Center, Nijmegen, Netherlands. Sera from age- and sex matched healthy controls were obtained from the Sanquin Bloedbank, Nijmegen, The Netherlands. Serum samples were stored at -80°C. All patients included in this study gave their informed consent and the study protocol was approved by the Medical Ethics Committee. In total, material of 28 patients was included in this study.

Microarray analysis

Total RNA was isolated from the synovial samples using the RNeasy kit for fibrous tissues (Qiagen, Venlo, Netherlands). 100 ng of total RNA was used for the preparation of biotinylated cRNA as described earlier[11] and hybridized to a U133Plus 2.0 oligonucleotide array (Affymetrix, Santa Clara, CA, USA), according to the Affymetrix Expression Analysis Technical Manual for two-cycle amplification. The arrays were scanned using the GeneChip Scanner (Affymetrix) and analyzed using

the GeneChip Operating Software version 1.4 (Affymetrix). Array normalization and expression value calculations were performed by the DNA-Chip analyzer version 1.3 (www.dchip.org) using the Invariant Set normalization and the Model-based method.[12] The RA synovium samples were obtained from the GEO database ([13], accession number GSE9027). The control samples were deposited under accession number GSE77298).

Cell culture

THP-1 monocytes were seeded in a 24-well plate at 3x10E5 cells/well for RNA analysis and multiplex assay or in a µClear white 96-well plate (Greiner Bio-one, Alphen a/d Rijn, Netherlands) at 5x10E4 cells/well for luciferase measurements in RPMI culture medium, supplemented with 10% FCS, 1 mM pyruvate and 1% pen/strep. Synovial cells were centrifuged and resuspended after red blood cell lysis and cultured at similar concentrations and conditions. All cell cultures were kept in humidified atmosphere at 37°C and 5% CO_2. The cells were stimulated with *E. Coli* lipopolysaccharide (LPS) (Invivogen, San Diego, CA, USA), Pam3CSK4 (Invivogen), rhIL-1β (R&D systems, Oxford, UK), rhTNFα (Abcam, Cambridge, UK) or rhIL-10 (Life Technologies Europe, Bleiswijk, Netherlands) at concentrations indicated in the text.

RNA isolation and qPCR

Total RNA from cells was isolated using TRI Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's recommendations and treated with DNase I (Invitrogen, Carlsbad, CA, USA) for 15 min. cDNA was synthesized by reverse transcription PCR using MLV Reverse transcriptase (Invitrogen) and oligo(dT) primers. The qPCR was performed using Power SYBR Green PCR master mix (Apllied Biosystems, Waltham, MA, USA) and the StepOnePlus Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. The reaction volume contained 5 µl SYBR master mix, 1 µl 2 µM forward primer, 1 µl 2 µM reverse primer and 3 µl cDNA template. Primer sequences are listed in Table S1. The PCR protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Plasmid cloning

For the production of lentiviral vectors, we made use of the third generation self-inactivating lentiviral (sin) vector. Cloning of the pRLL-cPPT-PGK-luc-PRE-SIN vector has been described previously.[14] The 535 bp proximal promoter of the *CXCL10* gene was isolated from purified human genomic DNA (Promega,

Madison, WI, USA) using Phusion polymerase (New England Biolabs, Ipswich, MA, USA), the forward primer TTTTGTCGACTCAAGGAGGACTGTCCAGGT and reverse primer TTTTGCTAGCGGTGCTGAGACTGGAGGTTC. The primers introduced restriction sites for SalI and NheI respectively. The promoter was first cloned into PCR-Script using the PCR-Script CAM cloning kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. SalI and NheI (New England Biolabs) were used to clone the CXCL10 promoter in the pRRL-cPPT-mcs-luc-PRE-SIN vector. The human *IL-10* transgene was isolated from cDNA obtained from primary human macrophages by nested PCR using forward primer AAAGAAGGCATGCACAGCTCA and reverse primer TGGAAGCTTCTGTTGGCTCC to obtain a 903 bp PCR nest. The 537 bp gene was subsequently isolated from the nest with forward primer GCTAGCGCCACCATGCACAGCTCAGCACTGCTC and reverse primer TCTAGATCAGTTTCGTATCTTCATTGTCATG, which introduced restriction sites for NheI and XbaI. NheI and XbaI (New England Biolabs) were used to replace the firefly luciferase gene in the pRLL-cPPT-CXCL10-luc-PRE-SIN by the therapeutic *IL-10* gene.

Lentivirus production

293T cells were seeded at density 1x10E5 cells/cm^2 in a T75 culture flask in DMEM medium, supplemented with 10% FCS, 1 mM pyruvate, 1% pen/strep and 0.01 mM cholesterol (Sigma) one day prior to the plasmid transfection. The cells were co-transfected with a calcium phosphate precipitate containing 5,3 µg sin vector, 4,0 µg MDL packaging plasmid, 3,5 µg VSV-G expression plasmid and 1,8 µg RSV-REV expression plasmid in medium without antibiotics. After 16h, the medium was replaced and new complete medium containing the produced virus was collected after 24h and 48h. The supernatant was passed through a 0.22 µm Stericup filter (Millipore, Bedford, MA, USA). Subsequently, 32 ml supernatant was layered on 4 ml 20% sucrose (Sigma) and centrifuged for 2h at 25k rpm in a Surespin 6x50ml swingout bucket rotor (Sorvall) in a Discovery 100WX ultra centrifuge (Sorvall). The viral vectors were resuspended in sterile PBS and stored at -80°C. Virus concentrations were determined using the INNOTEST HIV antigen mAb kit (Diasorin, Saluggia, Italy) and expressed as ng p24/µl. Viral transduction of cells was performed with 50 ng virus/5x10E4 cells in complete medium supplemented with 8 µg/ml polybrene (Sigma).

Luciferase measurements

The luciferase measurements were performed in 96-well white clear-bottom plates

(Greiner Bio-one) in total volumes of 50-60 µl using the BrightGlo luciferase assay system (Promega). 30 µl BrightGlo substrate was added to cells and light production was measured on a Lumistar luminometer (BMG, Offenburg, Germany). The values were corrected for the background signal and depicted as relative light units (RLU).

Multiplex Elisa assay

Cytokine and chemokine concentrations were determined by luminex multianalyte technology on the Bio-Plex 200 (Bio-Rad, Hercules, CA, USA) in combination with Bio-Plex pro human cytokine kits (Bio-Rad) according to the manufacturer's protocol. For IL-10, MCP-1, IL-6 and IL-8 measurements, the culture supernatants were first diluted 25x. Samples below the detection limit were set at the lowest measureable quantity to perform statistical analysis.

Statistical analysis

Statistical analysis was performed using the Student's t-test, 1-way ANOVA and 2-way ANOVA. Results are depicted as mean +/- SD and P-values < 0.05 were regarded as significant.

Results

Microarray analysis of RA synovium samples

Potential disease-regulated promoters can be obtained from endogenous promoters of genes upregulated during disease. Therefore, we analyzed 20 microarrays of RA patient synovium biopsies and compared the gene expression levels to 8 control synovium biopsies without arthritis. The criteria for candidate genes included at least 10-fold upregulation in RA with $P<0.01$ compared to control and a minimal average relative probe intensity of 500 (arbitrary units) in RA to find promoters of sufficient capacity. The genes that do not code for a transcribed mRNA were removed from the list. In addition, the immunoglobulin genes were removed, as their proximal promoters rely on B-cell-specific factors and are dependent on intronic enhancer elements.[15] 22 genes met these criteria and are summarized in Table 1.

The upregulation of the genes in the RA patient synovium was confirmed by qPCR on a subset of the biopsies **(Supp. Fig. 2, Table 1)**.

Table 1: List of genes upregulated in RA synovium, compared to control synovium

20 microarrays on RA synovium and 8 microarrays on control synovium. The average probe intensity was calculated with dchip using the invariant set normalization method and the model-based method. Depicted are the average intensity (+/- standard deviation) of the microarray sets, the index RA/HC, the P-value calculated by Student's t-test and the ΔΔCt values of the control PCR of RA biopsies compared to healthy controls. HC = healty control; RA = rheumatoid arthritis.

gene symbol	description	Average HC	Average RA	RA/HC	P-value	ΔΔCt
SPP1	secreted phosphoprotein 1	380,4 (346,9)	8773,6 (6754,4)	23,1	0,00001	8,0
SLAMF8	SLAM family member 8	71,9 (29,3)	889,9 (741,1)	12,4	0,00005	6,5
RGS1	regulator of G-protein signalling 1	49,6 (63,0)	598,0 (495,8)	12,0	0,00005	6,8
CCL18	chemokine (C-C motif) ligand 18	447,4 (214,5)	5279,3 (4523,3)	11,8	0,00007	7,8
CXCL13	chemokine (C-X-C motif) ligand 13	10,2 (3,5)	738,7 (746,3)	72,3	0,00017	12,0
IL4I1	interleukin 4 induced 1	52,8 (12,6)	677,3 (664,2)	12,8	0,00024	7,7
VSNL1	visinin-like 1	18,6 (4,5)	587,7 (629,3)	31,5	0,00035	2,3
TREM1	triggering receptor expressed on myeloid cells 1	295,7 (199,8)	3185,4 (3274,7)	10,8	0,00044	6,6
NMES1	normal mucosa of esophagus specific 1	39,3 (6,5)	808,5 (890,3)	20,6	0,00052	9,0
CXCL8	chemokine (C-X-C motif) ligand 8	126,5 (131,5)	1555,3 (1742,5)	12,3	0,00084	6,6
CXCL10	chemokine (C-X-C motif) ligand 10	92,5 (93,8)	1065,3 (1270,8)	11,5	0,00146	6,3
CXCL9	chemokine (C-X-C motif) ligand 9	202,2 (226,7)	2058,4 (2430,9)	10,2	0,00151	ND
MMP13	matrix metallopeptidase 13	41,2 (10,2)	930,0 (1192,5)	22,6	0,00175	6,7
CXCL6	chemokine (C-X-C motif) ligand 6	37,6 (21,9)	600,2 (778,8)	15,9	0,00221	5,4
CSN1S1	casein alpha s1	336,5 (626,5)	5763,7 (8196,4)	17,1	0,00412	6,2
FKBP11	FK506 binding protein 11, 19 kDa	63,0 (30,7)	732,6 (1023,1)	11,6	0,00434	6,5
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	26,8 (2,1)	550,5 (839,1)	20,5	0,00582	ND

SLAMF7	SLAM family member 7	36,7 (12,6)	527,5 (808,8)	14,4	0,00689	8,7
COL1A1	collagen, type I, alpha 1	45,2 (17,7)	608,9 (950,1)	13,5	0,00786	4,1
POU2AF1	POU domain, class 2, associating factor 1	50,7 (11,7)	1473,9 (2413,0)	29,1	0,00811	9,8
ADAMDEC1	ADAM-like, decysin 1	31,7 (27,8)	576,7 (924,8)	18,2	0,00818	9,6
IBSP	Integrin-binding sialoprotein	25,0 (16,0)	531,1 (873,4)	21,2	0,00897	4,7

We first determined if the gene upregulation in the joints was the result of pro-inflammatory signaling and if inflammation-sensitive cells can activate the gene promoters after stimulation *in vitro*. Human synovial biopsies were digested and stimulated for 6h with bacterial lipopolysaccharide (LPS), which activates the pro-inflammatory TLR4 signaling. Several genes that were selected from the microarrays were upregulated after stimulation, of which *CXCL10* showed the highest induction **(Fig. 1A)**. These results confirm that a pro-inflammatory stimulus can activate transcription of the genes and the promoters of these genes therefore might be applicable for disease-regulated gene therapy. Similar results were obtained in human THP-1 monocytes after stimulation with the synthetic lipopeptide Pam3CSK4, which triggers TLR2 signaling[16] **(Fig. 1B)**. Based on the microarray and the high inducibility in both synovial cells and THP1 cells, the *CXCL10* promoter was selected for the remainder of this study.

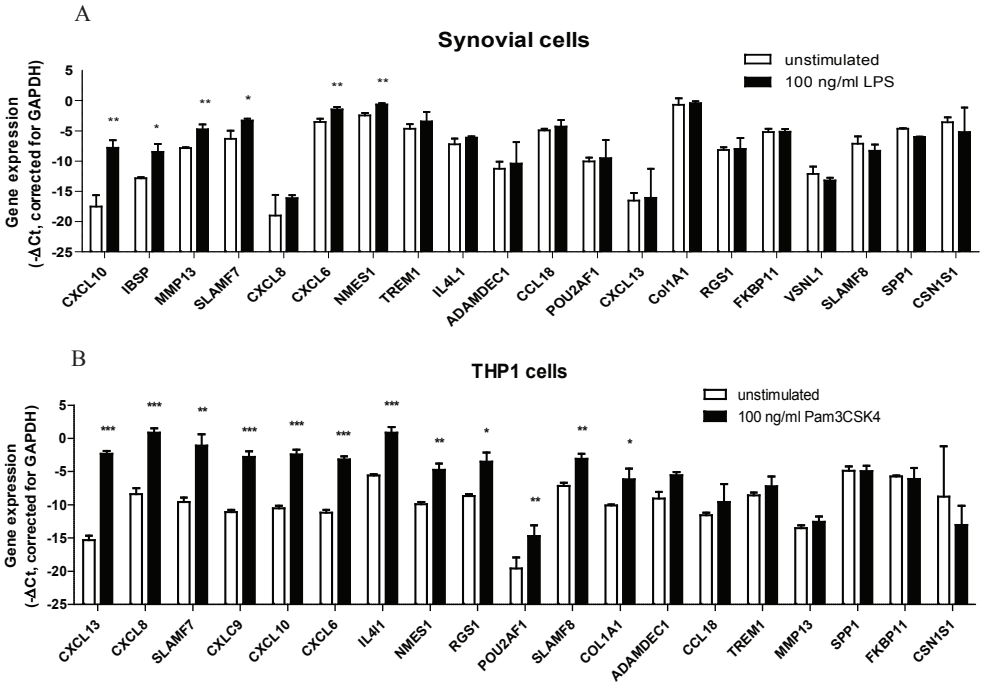


Figure 1: Upregulation of candidate genes in synovial cells and THP1 cells after TLR stimulation. (A) Gene expression levels in primary cell cultures of digested synovial biopsies from patient 21after 6h stimulation with 100 ng/ml LPS. Genes are sorted from left to right according to fold induction over unstimulated cells. **(B)** Gene expression levels in THP-1 cells stimulation with 100 ng/ml Pam3CSK4. Expression levels are depicted as threshold cycle (CT) +/- SD, corrected for GAPDH expression. Statistical analysis was performed by Student's t-test. * =P<0.05, ** =P<0.01, *** =P<0.001.

Promoter inducibility

The proximal promoter of the *CXCL10* gene was obtained from human cDNA and cloned into a lentiviral vector expressing the luciferase reporter gene. THP1 cells were transduced with the inducible reporter construct and stimulated with pro-inflammatory cytokines IL-1β, TNFα and TLR triggers LPS and Pam3CSK4 for 6h. The promoter showed a robust response to all stimuli **(Fig. 2A)**. In primary RA synovial cell culture, the isolated promoter was inducible by IL-1β, TNFα and LPS, but not by Pam3CSK4 **(Fig. 2B)**.

An important characteristic of disease-inducible therapy for RA is the net response of the promoter to the balance of pro- and anti-inflammatory proteins present in the patient. Therefore, we compared the *CXCL10* promoter response to serum from 63 RA patients to serum from 62 age- and sex matched healthy donors. The RA patient sera showed a stronger induction of the *CXCL10* promoter compared to healthy control sera **(Fig. 2C)**. This confirms that the inducible *CXCL10* promoter is more active in RA patients.

For the most accurate treatment of an RA patient, the inducible promoter activity should return to basal levels if the disease activity decreases and should be activated again if a new stimulus appears. Therefore, we compared the kinetics of the *CXCL10* promoter to the constitutive active PGK promoter at different time points after stimulation with TNFα. Indeed, the PGK-luciferase activity remained around basal levels for 96 hours **(Fig. 2D)**. The *CXCL10* promoter showed highest activity at 8-12h after the stimulation **(Fig. 2E)**, although the peak activity was still below PGK-level. As the TNFα was used and degraded by the cells,[17] the promoter activity decreased. Re-stimulation of the cells with TNFα 8h prior to the 96h time point resulted in a significant second induction of the promoter.

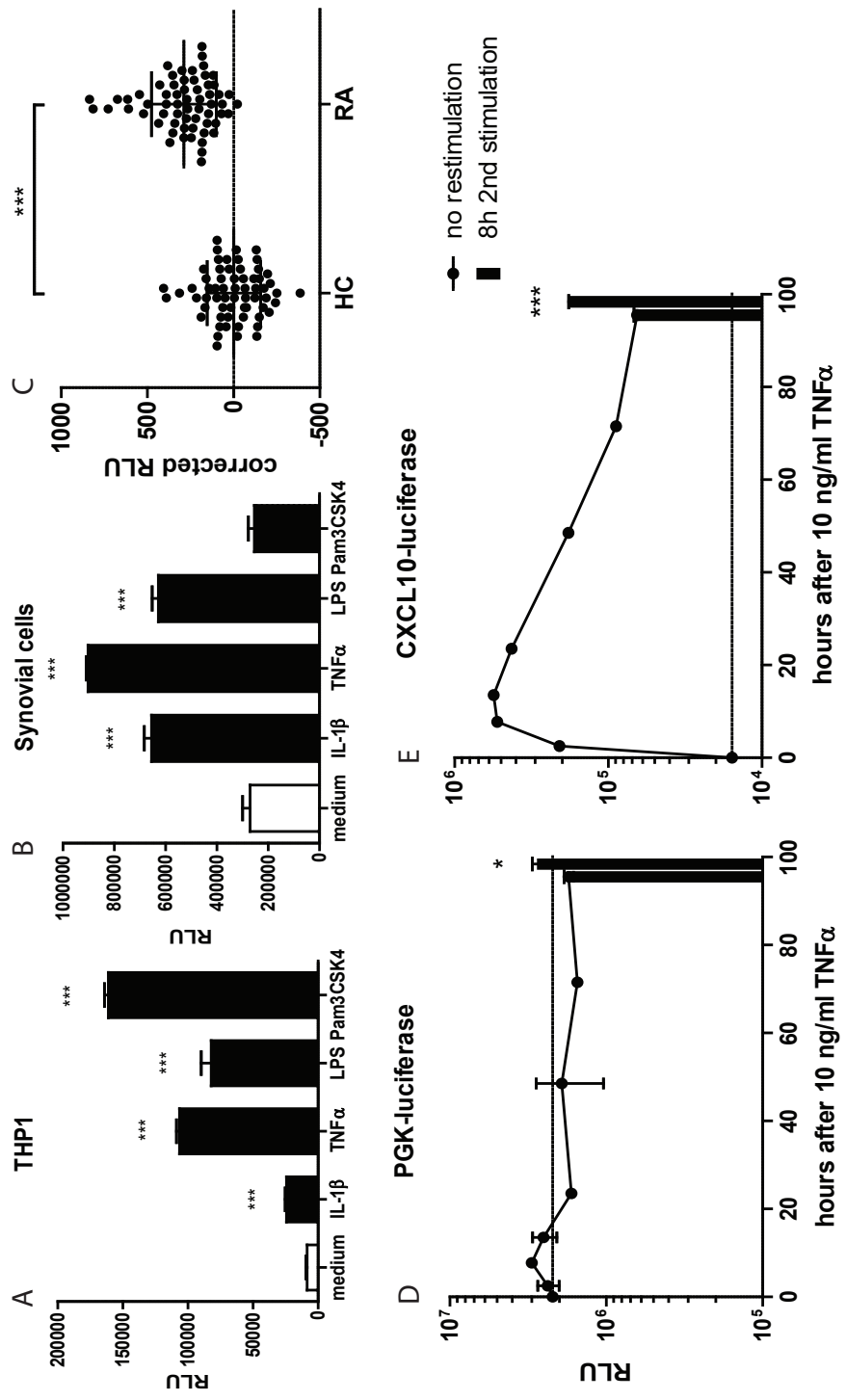

A
THP1
RLU
medium
IL-1β
TNFα
LPS
Pam3CSK4
B
Synovial cells
RLU
C
corrected RLU
HC
RA
D
PGK-luciferase
RLU
hours after 10 ng/ml TNFα
E
CXCL10-luciferase
no restimulation
8h 2nd stimulation
hours after 10 ng/ml TNFα

Figure 2: Inducibility of the *CXCL10* promoter in synovial cells and THP1 cells. (A) Relative light signal of *CXCL10* promoter-luciferase construct after stimulation with pro-inflammatory factors. THP1 cells were transduced with *CXCL10* promoter-luciferase lentiviral vector and stimulated for 6h with 10 ng/ml IL-1β, 10 ng/ml TNFα, 100 ng/ml LPS or 100 ng/ml Pam3CSK4. Light values are shown as relative light units (RLU) +/- SD. **(B)** *CXCL10* promoter-luciferase construct activation in primary RA synovial cell culture from patient 22. **(C)** *CXCL10* promoter-luciferase activation by RA patient serum. A stably transduced THP1 clone was stimulated with 10% RA patient serum or age- and sex-matched healthy control serum (HC). The light signal after 6h is shown as RLU, corrected for the average signal from the control group +/- SD. **(D,E)** THP-1 cells were transduced with lentiviral PGK-luciferase vector (D) or CXCL10-luciferase (E) and stimulated with 10 ng/ml TNFα. At multiple time points, the luciferase activity was determined. Light values are shown as relative light units (RLU) +/- SD. A second 10 ng/ml TNFα stimulation was provided at 88h, 8h prior to the last measurement. For statistical analysis, the re-stimulated sample was compared to the 96h time point without re-stimulation. Statistical analysis was performed by Student's t-test compared to the medium control. * =P<0.05, ** =P<0.01, *** =P<0.001.

Recombinant human IL-10 effects on cytokine production

The therapeutic potential for IL-10 was assessed using recombinant human IL-10 (rhIL-10). Primary RA synovial cell cultures of 3 patients were stimulated for 6h with LPS with or without the addition of rhIL-10. Despite differences in absolute quantity between patients, the production of TNFα was increased after LPS stimulation and could be significantly reduced with rhIL-10 **(Fig. 3A)**. To determine the effects of the cytokines produced by the synovial cells on the *CXCL10* promoter, THP1 cells transduced with CXCL10prom-luciferase were stimulated with the synovial cell culture supernatant of one of the patients from Figure 4A (**Fig. 3B**, closed bars). To test the effects of residual LPS and IL-10 that might be left in the supernatant from the synovial cells, LPS and rhIL-10 effects were first tested separately (open bars). No direct activation of the *CXCL10* promoter by rhIL-10 was observed and LPS showed some activation of the *CXCL10* promoter. However, the cell culture supernatant from LPS-stimulated synovial cells showed a stronger induction compared to LPS alone. If the synovial cells were treated with rhIL-10, the capacity of its supernatant to activate the *CXCL10* promoter was significantly reduced. This shows that activated synovial cells produce the required factors to activate the *CXCL10* promoter and that IL-10 can diminish this production. All conditions were also tested in the presence of the specific TNFα-inhibitor Etanercept. In the presence of Etanercept, the *CXCL10* promoter induction by synovial cell supernatant was significantly lower, indicating that TNFα produced by the stimulated synovial cell culture is the most important trigger for *CXCL10* promoter activation in this setting.

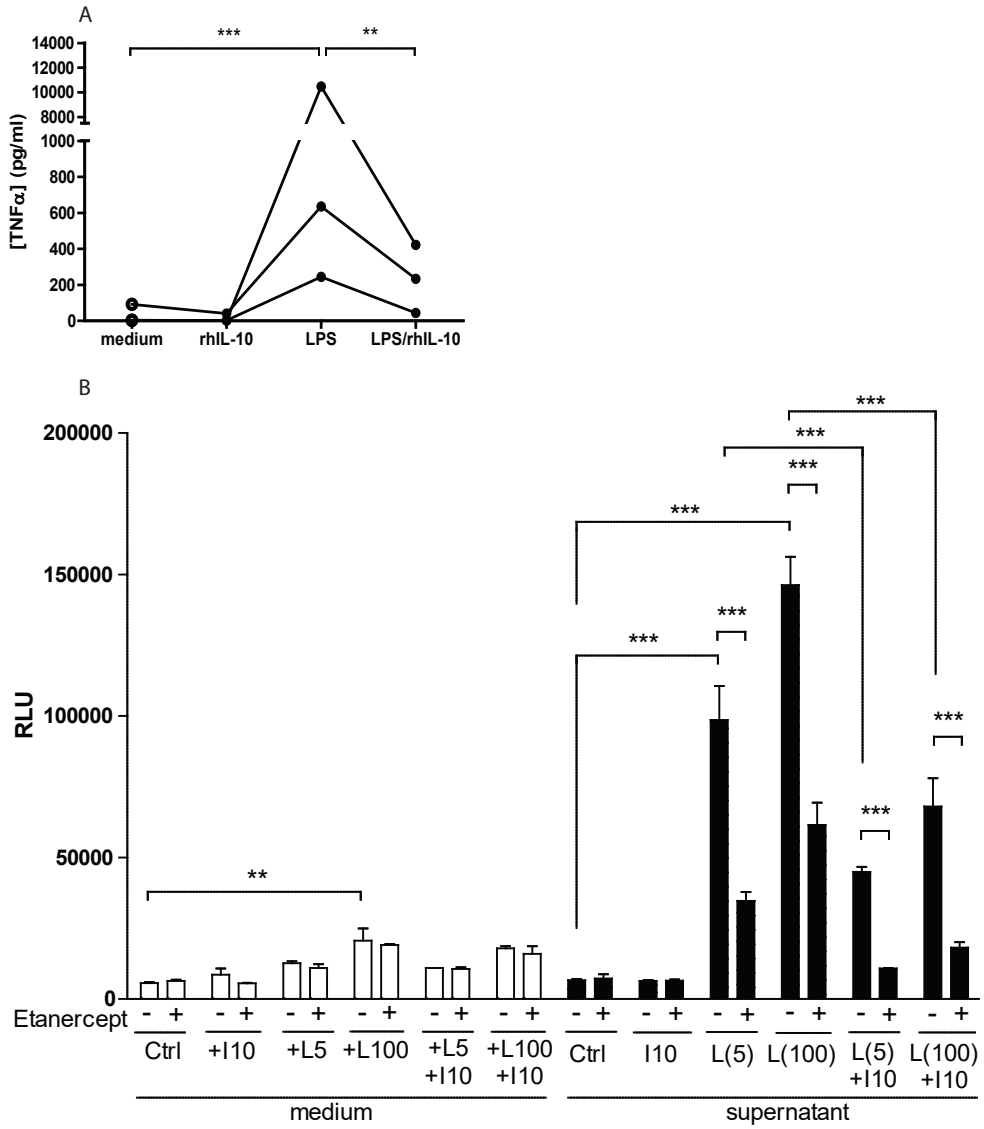


Figure 3: TNFα levels in the supernatant of stimulated synovial cells and the activation of the CXL10p-luciferase by the supernatant. (A) Synovial biopsies from patient 23, 24 and 25 were digested using Liberase and passed through a cell strainer. After red blood cell lysis, cells were seeded and allowed to attach and recover. Subsequently, the synovial cell suspension was stimulated for 6h with 5 ng/ml LPS and 20 ng/ml recombinant human IL-10. The cell culture medium was removed and the produced TNFα was measured using multiplex assay. **(B)** THP1 cells stably transduced with the CXCL10p-luciferase reporter lentivirus were stimulated for 6h with medium containing 20 ng/ml

recombinant IL-10 (+I10), 5 ng/ml LPS (+L5), 100 ng/ml LPS (+L100) or a combination (open bars). In addition, the cells were stimulated with the cell culture supernatant obtained from Figure 4A (closed bars) that might contain residual IL-10 (I10) or LPS (L(5) and L(100)). All samples were tested in the presence and absence of 50 ng/ml TNFα-inhibitor Etanercept. Light values are shown as relative light units (RLU) +/- SD. Results were similar with supernatants from all three patients (data not shown). Statistical analysis between subgroups of different stimulations was performed by 2-way ANOVA and other comparisons were performed by Student's t-test. ** =P<0.01, *** =P<0.001.

Effects of CXCL10-IL10 lentivirus on cytokine production

The potential of the *CXCL10* promoter to produce therapeutic quantities of IL-10 was tested by replacing the luciferase reporter transgene by the coding region of the human *IL-10* gene (CXCL10p-IL10). THP-1 cells transduced with PGK-luciferase control lentiviral vector produced low quantities of IL-10 **(Fig. 4A)**. After transduction with CXCL10p-IL10, the basal activation of the of the *CXCL10* promoter already resulted in increased IL-10 levels. Stimulation of the transduced cells with Pam3CSK4 caused a significant increase in production of IL-10. Pam3CSK4-stimulated THP-1 cells with control virus showed a marked increase in production of inflammatory cytokines MCP-1, IL-6 and IL-8 **(Fig. 4B-D)**. The release of these cytokines was inhibited by the CXCL10p-IL10 lentivirus, which shows the anti-inflammatory potential of the CXCL10p-IL10 treatment.

Subsequently, the CXCL10p-IL10 lentivirus was tested in synovial cell cultures of three patient donors. After transduction with control virus or CXCL10p-IL10, the cells were stimulated with 5 or 100 ng/ml LPS. Only minor quantities of IL-10 were produced by cells with the control virus **(Fig. 5A)**. After transduction with the CXCL10p-IL10 lentivirus, basal production levels of IL-10 were observed, which significantly increased after LPS stimulation in all patients, although the magnitude varied between patients. LPS triggered the release of TNFα, which was reduced by the CXCL10p-IL10 treatment of the cells **(Fig. 5B)**. With the exception of the third donor, the same effects were also observed for IL-1β. In the third donor, IL-1β levels remained low after stimulation with LPS **(Fig. 5C)**. The production of MCP-1, IL-8 and IL-6 were also decreased in cells transduced with CXL10p-IL10, although the decrease was not significant for most samples **(Supp. Fig. 3)**. These results show that in the synovial cells of the patient, the CXCL10p-IL10 gene therapy can provide inducible expression of therapeutic levels of IL-10 that can suppress the production of pro-inflammatory cytokines.

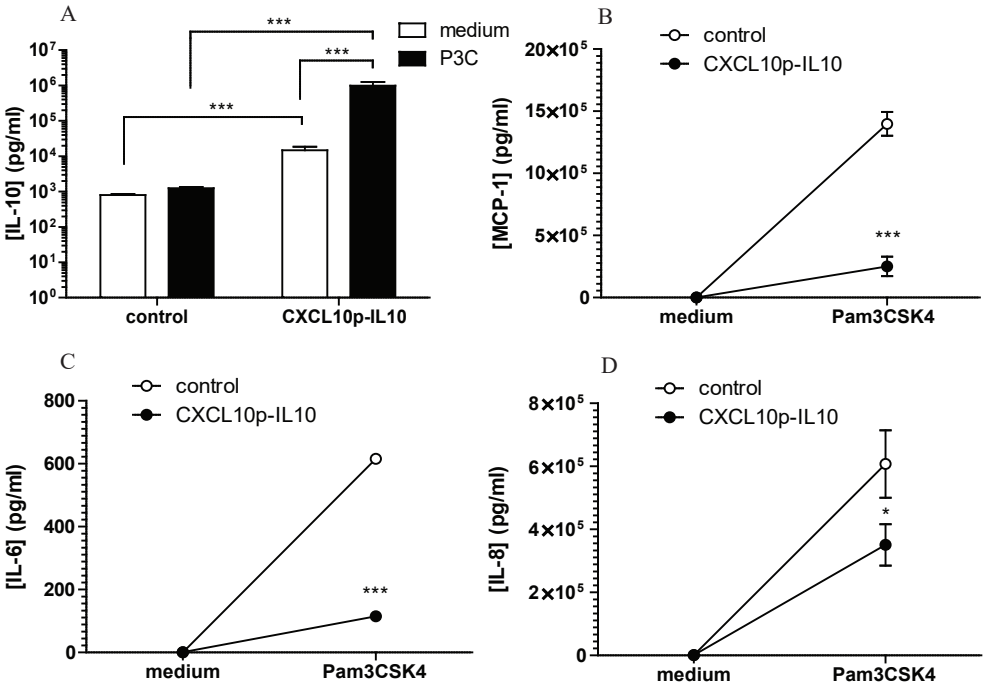
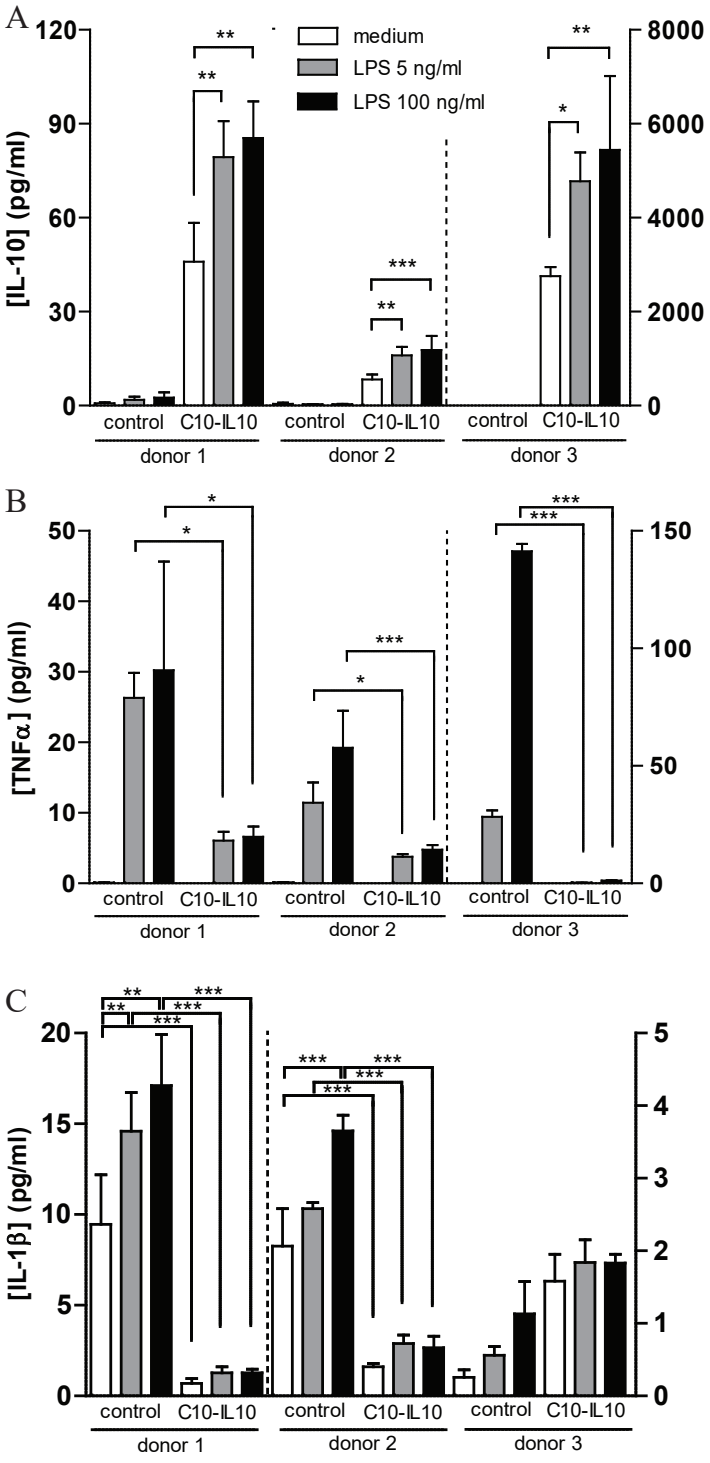


Figure 4: Production **and effects of IL-10 in CXCL10p-IL10 transduced THP1-cells. (A)** THP-1 cells were transduced with lentiviral vectors containing PGK-luciferase or CXCL10p-IL10. After 48h, the cells were stimulated with medium control or with 100 ng/ml Pam3CSK4 for 24h. IL-10 production was measured in the culture supernatant by multiplex assay. Statistical analysis was performed by 1-way ANOVA. **(B-D)** THP-1 cell culture supernatant concentrations of MCP-1 **(B)**, IL-6 **(C)** and IL-8 **(D)**. Statistical analysis was performed by Student's t-test, compared to the stimulated control cells. * =P<0.05, *** =P<0.001.

Figure 5: Production and effects of IL-10 on TNFα and IL-1β in CXCL10p-IL10 transduced synovial cells. Synovial tissue biopsies from patient 26, 27 and 28 were digested and cells were transduced with lentiviral PGK-luciferase (control) or lentiviral CXCL10p-IL10. After a minimum of 48h after transduction, the cells were stimulated for 24h with 5ng/ml LPS or 100 ng/ml LPS. The supernatant was collected and the concentrations of IL-10 **(A)**, TNFα **(B)** and IL-1β **(C)** were measured by multiplex assay. The cytokine concentration of samples left of the dotted line are shown on the left y-axis and the samples on the right of the dotted line are depicted on the right y-axis. Statistical analysis was performed by 1-way ANOVA. * =P<0.05, ** =P<0.01, *** =P<0.001.


A
B
C
medium
LPS 5 ng/ml
LPS 100 ng/ml
[IL-10] (pg/ml)
[TNFα] (pg/ml)
[IL-1β] (pg/ml)
control
C10-IL10
donor 1
donor 2
donor 3

Discussion

In this study we showed that the promoter from the *CXCL10* gene is inducible by pro-inflammatory TLR triggers and cytokines. In human THP-1 monocytes, the promoter showed a significant induction by RA patient serum, compared to healthy control serum. Using the CXCL10p-IL10 lentivirus in LPS-stimulated cells from RA patient biopsies, therapeutic levels of anti-inflammatory IL-10 were produced that could reduce the release of pro-inflammatory cytokines TNFα, IL-1β, IL-6, IL-8 and MCP-1.

The promoters of genes that were upregulated in RA synovium microarrays are the possible candidates for disease-regulated therapy. We first determined if the gene upregulation was the result of pro-inflammatory signaling rather than an altered cell composition in RA synovium by using broad TLR triggers which activate the major inflammatory transcription factors.[18] TLRs are important players in RA and can be triggered by damage-associated molecular patterns (DAMPs) present in the RA joint.[19] A TLR2 trigger was used for THP1 cells, because TLR2 shows the highest expression in THP1 cell.[20] In contrast, TLR2 is not uniformly expressed on synovial fibroblasts and is upregulated after stimulation.[21,22] Therefore, the synovial cell cultures were stimulated with a TLR4 ligand. Based on the obtained data, the promoter of the *CXCL10* gene was selected. In accordance with our results, previous studies have shown increased levels of CXCL10 in synovial fluid of RA patients.[23]

CXCL10 is a C-X-C motif ligand chemokine, responsible for the recruitment of several immune cell types into the inflamed joint.[24] The isolated region of the *CXCL10* promoter contains predicted binding sites for immunoregulatory transcription factors C/EBPβ, NF-κB, AP-1 and an interferon-stimulated response element (ISRE).[25] Point mutations in the NF-κB and the ISRE sites resulted in reduced promoter activity after stimulation with TNFα and IFNγ or by TLR triggers in hepatocytes.[26] Interestingly, promoter constructs with mutations in the AP-1 or C/EBPβ binding sites showed increased activity upon stimulation, indicating orchestration of the promoter activity by multiple factors.

The *CXCL13* gene, also known as B-lymphocyte chemoattractant, showed the highest upregulation in the RA microarrays and in stimulated THP-1 cells. In mice, the promoter of *CXCL13* contains a TATA-box and transcription factor binding sites.[27] In humans this region is conserved, but located in a large intron in the 5'UTR. We isolated both the proximal promoter region from the human transcription start site and the first intron, but we could not observe an upregulation of either promoter activity after stimulation (data not shown). In addition, we did not find an upregulation of endogenous *CXCL13* in Pam3CSK4-stimulated synovial cells.

During RA pathogenesis, cells in the joint produce chemokines and cytokines to induce pannus formation and attract different immune cells to the joint, including neutrophils, monocytes, T-cells and B-cells.[28,29] As a consequence, the synovial cell cultures after digestion of the synovium are of heterogeneous composition with patient-to-patient variation. This results in differences in cytokine production levels. For example, Il-1β has been suggested to be predominantly produced by activated monocytes and macrophages.[30] The third donor in Figure 5 showed the highest TNFα production upon stimulation, but only a very low production of IL-1β, which might reflect a low abundance of monocytes.

In general, the cells in the synovium of RA patients have an inflammatory phenotype, which has also been observed in microarrays. Compared to healthy controls, the signaling networks associated with upregulated genes in RA include inflammatory response, T-cell activation and apoptosis.[31] As a result, the cytokine concentrations are increased in the synovial fluid of RA patients.[32] The inflammatory phenotype of the synovial cells in this study is reflected by the basal production of IL-1β (Figure 5C) and IL-10 after CXCL10p-IL10 transduction (Figure 6A) and by a relatively high basal activity of the *CXCL10* promoter in RA synovial cells (Figure 2B).

To avoid effects of the basal activation state on the *CXCL10* promoter, THP-1 cells were used to study promoter activation. THP-1 cells are human monocytes which are responsive to multiple TLR ligands and cytokines and can subsequently activate different inflammatory pathways.[33] In addition, gene expression analysis of peripheral blood in RA patients showed an enrichment in monocyte-specific transcripts,[34] indicating that THP-1 is a sensitive cell model relevant to RA. Indeed, the *CXCL10* promoter in THP-1 cells showed a strong induction by the cytokines produced by stimulated synovial cells. In addition, the responsiveness of the *CXCL10* promoter in a monocyte cell line to RA serum factors might enable the use of the promoter to treat systemic components of the disease, like atherosclerosis and autoimmunity.

Patients with inherited deficiencies in IL-10 signaling are rare, but suffer from early-onset inflammatory bowel disease (IBD).[35] In addition, some studies found that the frequency of a polymorphism associated with low serum levels of IL-10 was increased in RA patients.[36,37] Currently, research is focusing on obtaining long-term active IL-10 targeted to the inflamed joint both by disease-responsive gene therapy and by fusing IL-10 to homing antibodies[38] to provide regulated suppression of the inflammation.

In conclusion, we have shown the temporal and spatial activation of the *CXCL10* promoter, which was used to provide inducible expression of the anti-

inflammatory IL-10. The vector was capable of reducing the release of pro-inflammatory cytokines by RA synovial cells and might therefore be suited to provide local and disease-responsive gene therapy in RA patients.

Acknowledgements

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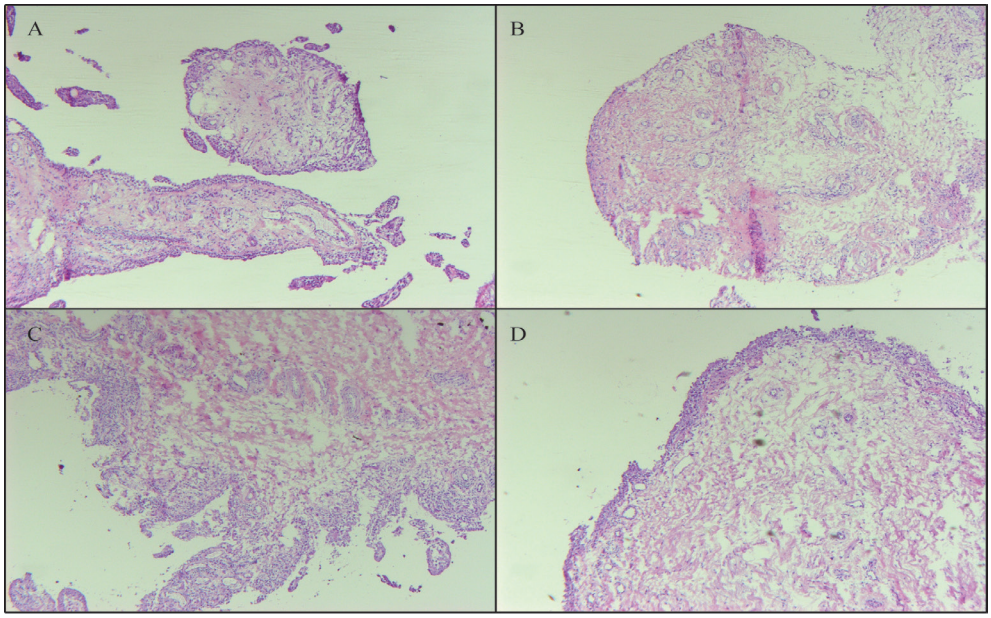
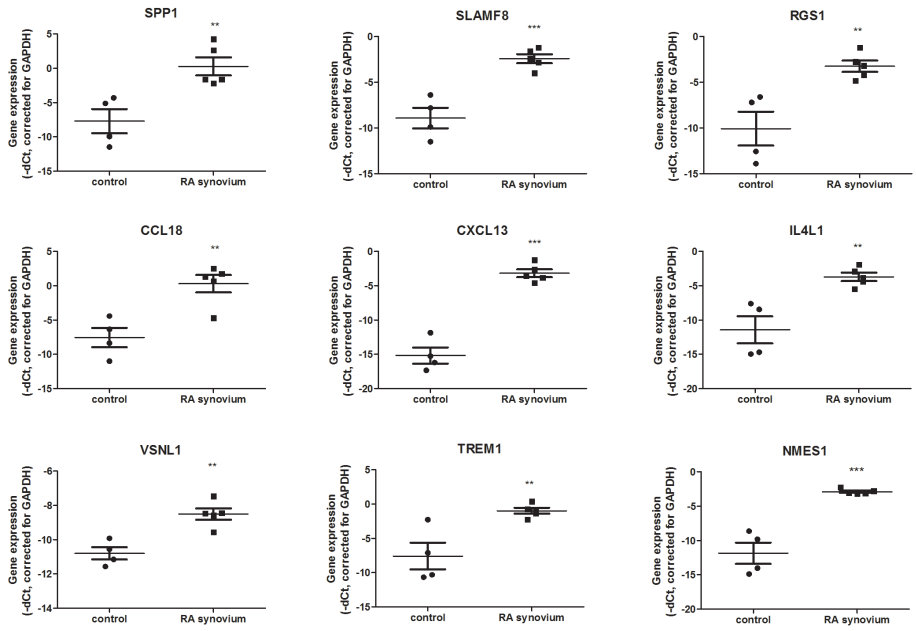
Supplementary information

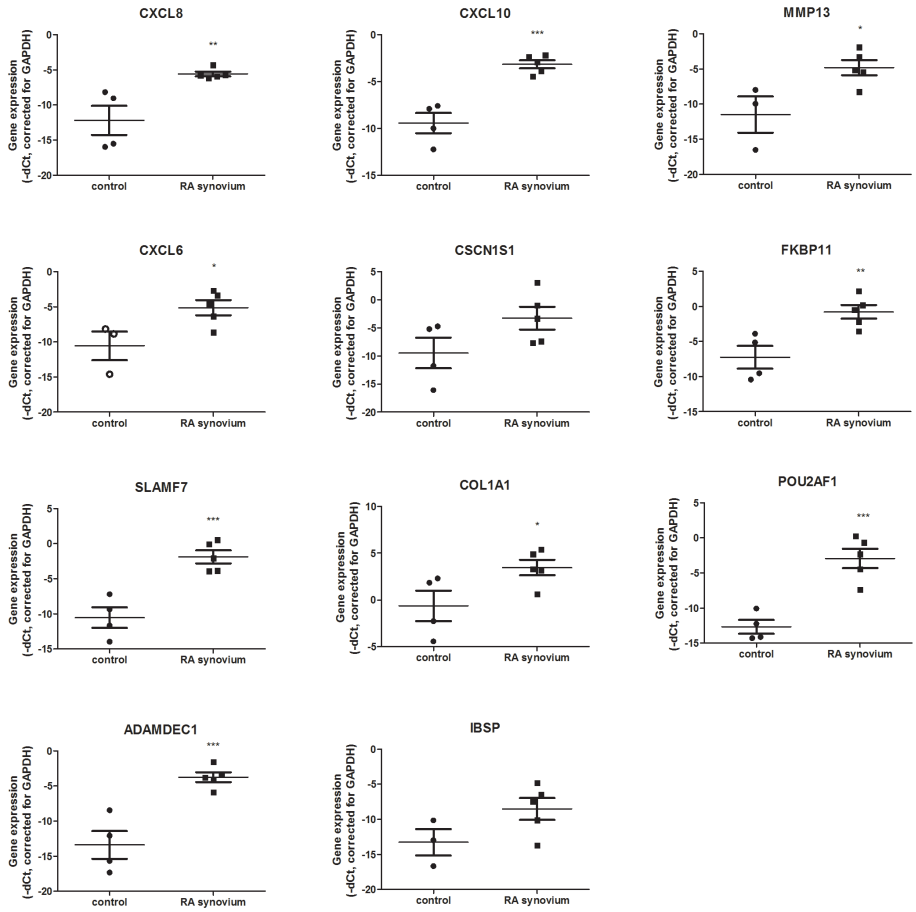
Table S1: List of primer sequences for qPCR analysis

Gene symbol	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTGAGC
CXCL13	CTCTGCTTCTCATGCTGCTG	CAGCTTGAGGGTCCACACAC
VSNL1	CCAATGGGGAGGCTAAATC	GAAGTCAATGGTGCCGTC
POU2AF1	CAATGTCACGACAAGAAGCT	CACGGGAAATAGGTGAGGG
SPP1	CTCCTAGGCATCACCTGTG	GGGTATTTGTTGTAAAGCTGCT
MMP13	ATTAAGGAGCATGGCGACTTCT	CCCAGGAGGAAAAGCATGAG
IBSP	GCAGTAGTGACTCATCCGA	CTCCTTCATTTGAAGTCTCCTC
NMES1	AGGAAGGAACTCATTCCCT	CCAAAGAGAATACACAGCGA
TNFRSF17	CGACTCTGACCATTGCTTTCC	AAGCAGCTGGCAGGCTCTT
ADAMDEC1	TCAAACTCAAGCAATAGCCA	TGTCTGGTTGTTCTTGATCTC
CSN1S1	AGGCTCTGATAACCATGAGG	GTTTCTCTGCCTGTTCATACC
CXCL6	AGAGCTGCGTTGCACTTGTT	GCAGTTTACCAATCGTTTTGGGG
SLAMF7	TCCACTGTGGAAATACCGA	GGCAAATAGCCTTGGTGTG
COL1A1	AGATCGAGAACATCCGGAG	AGTACTCTCCACTCTTCCAG
IL4I1	CTCAAAGACCTCAAGGCAC	CCCGAGAAGATATTCCAAGAG
SLAMF8	CTCAAGGTGTACGATGCAG	GTTATTTCGCTGATGTTGGG
CXCL8	AGAAGTTTTTGAAGAGGGCTGAGA	AGTTTCACTGGCATCTTCACTGATT
RGS1	ACTCATTCACTTCTAGACGAC	GGTATGCTTTCATATCCATTCC
CCL18	GGTGTCATCCTCCTAACCA	GTCGCTGATGTATTTCTGGAC
FKBP11	GATTCCAGGTCTGGAGCAG	CATAGGCCAAGTGAGAAGGA
CXCL10	ATTTGCTGCCTTATCTTTCTG	GACATCTCTTCTCACCCTTCT
TREM1	TTGTCTCAGAACTCCGAGC	CATTTCACATCCAGGGTCTG
CXCL9	GGAGTGCAAGGAACCCCAGTA	GAGAAACAGGTCAGCCAAAAG

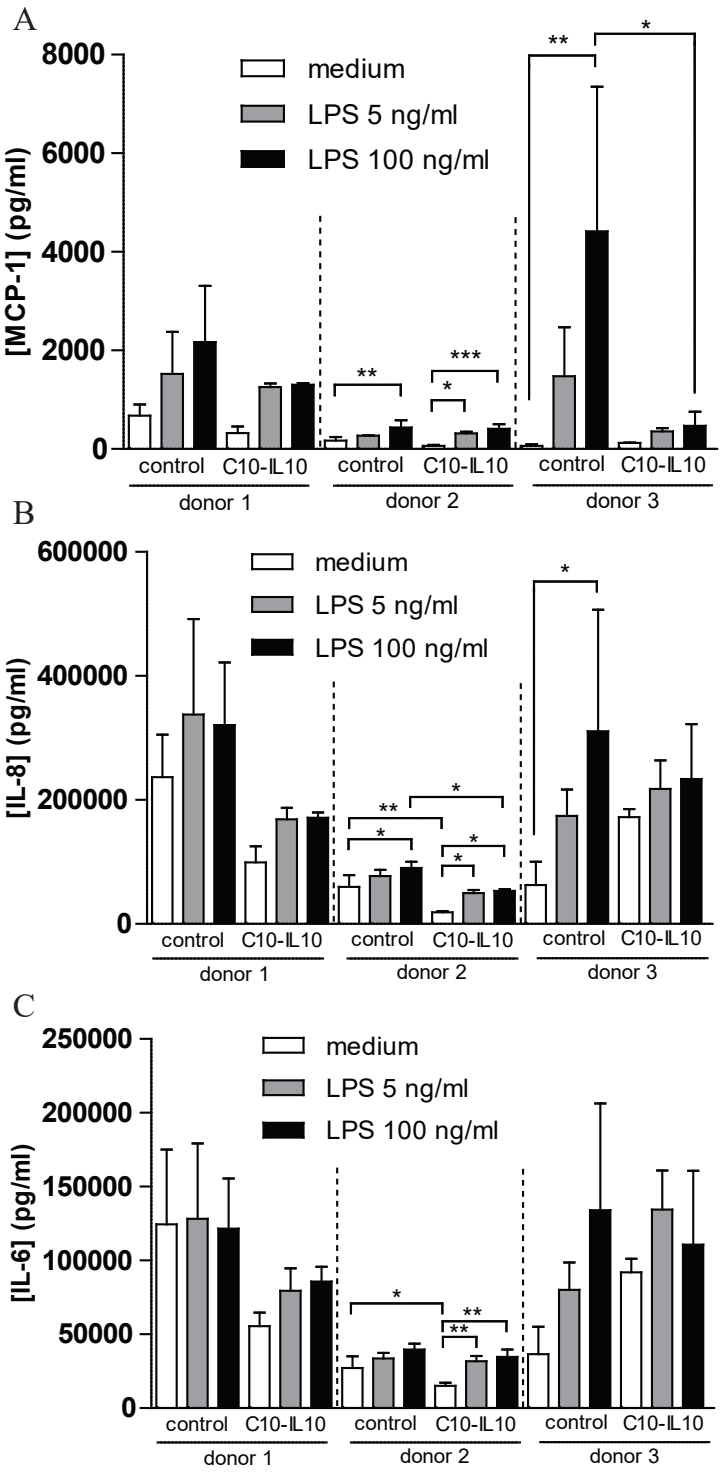


Supplemental Figure 1: Examples of histological sections of the synovium samples used in this study from patients 23, 24, 26 and 27. 7 µm cryosections were stained with haematoxylin and eosin.





Supplemental Figure 2: Gene expression levels of genes upregulated in RA in synovium biopsies. n=4 and n=5 samples per group were measured and the expression levels are depicted as threshold cycle (CT) +/-SD, corrected for GAPDH expression. Statistical analysis was performed by Student's t-test. * =P<0.05, ** =P<0.01.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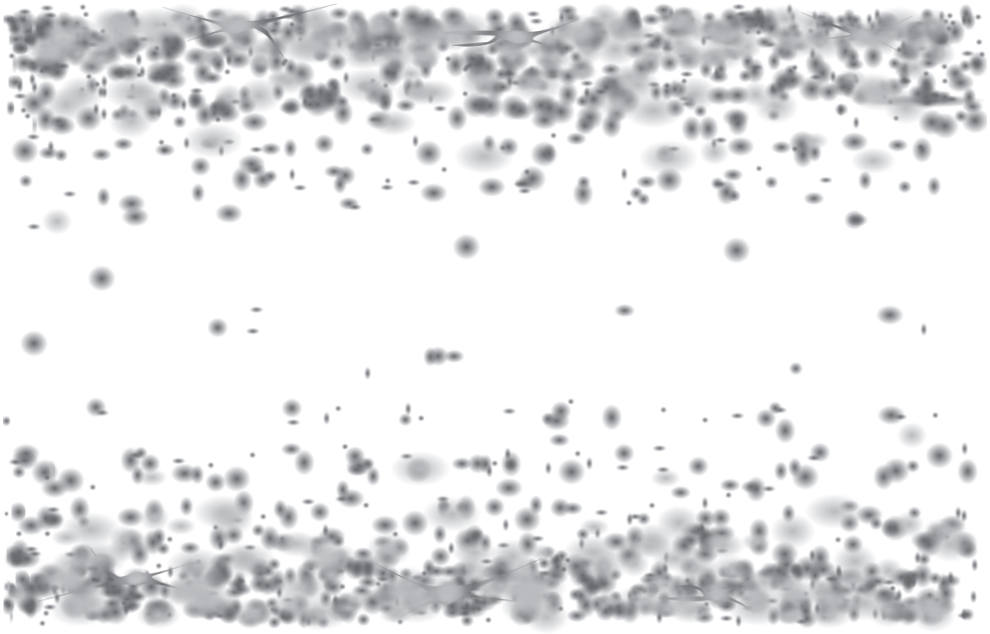
6000
4000
2000
0
medium
LPS 5 ng/ml
LPS 100 ng/ml
control
C10-IL10
donor 1
donor 2
donor 3
B
[IL-8] (pg/ml)
600000
400000
200000
0
C
[IL-6] (pg/ml)
250000
200000
150000
100000
50000
0

Supplemental Figure 3: Production and effects of IL-10 on MCP-1, IL-8 and IL-6 in CXCL10p-IL10 transduced synovial cells. Synovial tissue biopsies from patient 26, 27 and 28 were digested and cells were transduced with lentiviral PGK-luciferase (control) or lentiviral CXCL10p-IL10. After a minimum of 48h after transduction, the cells were stimulated for 24h with 5ng/ml LPS or 100 ng/ml LPS. The supernatant was collected and the concentrations of MCP-1 **(A)**, IL-8 **(B)** and IL-6 **(C)** were measured by multiplex assay. Statistical analysis was performed by 1-way ANOVA. * =P<0.05, ** =P<0.01, *** =P<0.001.



Chapter 7

Suppression of the inflammatory response by disease-inducible interleukin-10 gene therapy in a three-dimensional micromass model of the human synovial membrane.

Mathijs G.A. Broeren, Marieke de Vries, Miranda B. Bennink, Onno J. Arntz, Peter L.E.M. van Lent, Peter M. van der Kraan, Wim B. van den Berg, Frank H.J. van den Hoogen, Marije I. Koenders, Fons A.J. van de Loo

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Background:
Gene therapy has the potential to provide long-term production of therapeutic proteins in the joints of osteoarthritis (OA) patients. The objective of this study was to analyse the therapeutic potential of disease-inducible expression of anti-inflammatory interleukin-10 (IL-10) in the 3-dimensional micromass model of the human synovial membrane.

Methods:
Synovial tissue samples from OA patients were digested and the cells were mixed with Matrigel to obtain 3D micromasses. The *CXCL10* promoter combined with the firefly luciferase reporter in a lentiviral vector was used to determine the response of the *CXCL10* promoter to tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and lipopolysaccharide (LPS). The effects of recombinant IL-10 on gene expression were determined by quantative PCR. The production of IL-10 from the CXCL10p-IL10 vector and the effects on pro-inflammatory cytokine production were assessed by multiplex ELISA.

Results:
Micromasses made from whole synovial membrane cell suspensions form a distinct surface composition containing macrophage and fibroblast-like synoviocytes thus mimicking the synovial lining. This lining can be transduced by lentiviruses and allow CXCL-10 promoter-regulated transgene expression. Adequate amounts of IL-10 transgene were produced after stimulation with pro-inflammatory factors able to reduce the production of synovial IL-1β and IL-6.

Conclusions:
Synovial micromasses are a suitable model to test disease-regulated gene therapy approaches and the CXCL10p-IL10 vector might be a good candidate to decrease the inflammatory response implicated in the pathogenesis of OA.

Background

Osteoarthritis (OA) is the most common joint disease and no adequate disease-modifying treatments are available yet.[1] Although the exact etiology of OA is still unclear and may be dependent on multiple risk factors, there is increasing evidence that in many OA patients inflammation is involved in the pathogenesis.[2] IL-1β and TNFα are key pro-inflammatory cytokines, primarily produced by macrophages that infiltrate the synovium during inflammatory OA.[3] These cytokines trigger the release of other cytokines and matrix-degrading enzymes from resident articular cells, including the fibroblast-like synoviocytes (FLS) that damage the articular cartilage. This results in the release of damage-associated molecular patterns (DAMPs) with pro-inflammatory properties, potentially creating a positive vicious circle of inflammation and damage.[4]

Several strategies have been explored to treat inflammation in the OA joint, including biological therapies that were developed for the treatment of rheumatoid arthritis (RA). However, the therapeutic effects are disappointing.[5] A possible explanation might be the relative strong contribution of systemic factors in RA. In contrast, OA is considered primarily to be a local process and systemically injected therapeutics may not reach the target joints in sufficient amounts. Intra-articular injections are feasible but proteins injected in the joint are rapidly cleared and consequently the invasive injections have to be repeated increasing the discomfort for patients.[6] These considerations ask for the development of a different strategy to obtain long-term drug effects in the OA joint. Local gene therapy can be a suitable approach and might even be used to express proteins with a short half-life like interleukin-10 (IL-10), which is a potent anti-inflammatory cytokine.[7]

Previous studies with IL-10 gene therapy in experimental models of rheumatoid arthritis have explored the use of inducible promoters to drive the expression of IL-10.[8-10] These promoters contain binding sites for transcription factors that are activated during active disease, which might reduce side effects of continuous IL-10 exposure as observed in patients suffering from chronic active Crohn's disease. These patients received daily injections of high dose IL-10 and showed a drop in hemoglobin levels.[11] We recently adapted the inducible IL-10 gene therapy to human synovial cells.[12] For this purpose, the promoter from the *CXCL10* gene was selected based on microarray analysis of RA synovium. The inflammatory chemokine CXCL10 protein concentration has also been found to be significantly upregulated in the synovial fluid and in the serum of OA patients compared to healthy controls.[13,14] Because CXCL10 can be expressed from multiple cell types, no selective expression is expected from

the vector.[15] CXCL10 expression is associated with OA-related disease processes, including inflammation and osteoclastogenesis,[15] which indicates that *CXCL10* promoter-driven expression of IL-10 might be a viable option for the treatment of OA. In addition, the OA synovium could be more sensitive to IL-10 therapy, because of relatively high expression of the IL-10 receptor α chain as compared to RA.[16]

The CXCL10p-IL10 gene therapy approach showed promising results in synovial cell suspensions. Based on the known IL-10 effects we postulate that local IL-10 gene therapy would be efficacious at the early stage of OA when synovitis is developing and before irreversible fibrotic changes occur. In this study, we determined the inflammatory response and anti-inflammatory potential of the CXCL10p-IL10 lentiviral vector in the three-dimensional (3D) micromass synovial membrane model. In a 3D culture model, the cell-matrix and cell-cell interactions are more biologically relevant, providing a more predictive system for the *in vivo* situation, compared to classic 2D culture.[17] Synovial micromasses were generated from primary synovial cells isolated from OA patients by digestion, containing both FLS and macrophage-like synoviocytes (MLS). The micromasses were transduced after establishment of a synovial lining layer and the *CXCL10* promoter was responsive to lipopolysaccharide (LPS), TNFα and IL-1β. The activated promoter could provide therapeutic quantities of IL-10, which reduced the release of IL-1β and IL-6. These results show that the CXCL10p-IL10 vector can provide self-regulated inhibition of the inflammatory response in a synovial membrane model.

Materials and methods

Patient material

Synovial osteoarthritis (OA) tissue samples were obtained during joint replacement surgery from the department of Orthopedics (Radboud university medical center, Nijmegen, The Netherlands). Patients gave their informed consent and protocols were approved by the medical ethics committee. In total, synovium of 12 OA patients was included in this study. The micromasses shown in Figure 1A-D were derived from a patient diagnosed with RA. Before processing, representative samples were embedded in Tissue-Tek O.C.T. (Sakura, Alphen a/d Rijn, The Netherlands). 7 µm cryosections were cut using the Cryostar NX70 (Thermo Scientific, Waltham, MA, USA) and stained for hematoxylin and eosin (HE) to confirm that the tissue samples contained a synovial lining. **Fig. S1** contains HE images of 12 patients.

The synovial samples were digested using 50 µg/ml Liberase TM (Roche, Basel, Switzerland) for 1h at 37°C in RPMI culture medium without supplementations. The digestion was stopped by adding 10% fetal calf serum (FCS). Subsequently, the synovial cells were passed through a 70 µm cell strainer (Corning, NY, USA) and centrifuged. All cell centrifugations were performed for 5 min at 1500 rpm/423 g in a Heraeus Megafuge 16R (Thermo Scientific). Red blood cells were lysed for 2 min at RT using 4 ml RBC lysis buffer (155 nM NH_4Cl, 12 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). The lysis reaction was quenched by adding 6 ml RPMI culture medium, supplemented with 10% FCS and 1 mM pyruvate and 1% P/S.

Micromass production and culture

For micromass construction, the synovial cell suspensions were centrifuged and cell pellets were dissolved in ice-cold Matrigel (Corning) at an average density of 2x10E7 cells/ml. Using cooled pipette tips, 25 µl droplets were placed in 24-well culture plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) or conical 12 ml tubes (Greiner Bio-one), which were coated with poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich, Zwijndrecht, The Netherlands). After 30 minutes gelation at 37°C, 500 µl RPMI culture medium, supplemented with 10% heat-inactivated FCS, 1 mM pyruvate and 1% P/S was added. Medium was replaced twice weekly. All cell cultures were kept in humidified atmosphere at 37°C and 5% CO_2. The micromasses were stimulated with *E. coli* lipopolysaccharide (LPS) (Invivogen, San Diego, CA, USA), recombinant human TNFα (Abcam, Cambridge, UK), recombinant human IL-1β (R&D systems, Oxford, UK) and recombinant human IL-10 (Life Technologies Europe, Bleiswijk, The Netherlands) at concentrations and timing as indicated in the text.

Micromass immunohistochemistry

For immunohistochemical analysis, micromasses were fixated for 2h in 2% paraformaldehyde in phosphate-buffered saline (PBS)/1mM $CaCl_2$, dehydrated and embedded in paraffin. 7 µm sections were deparaffinized, rehydrated and incubated with antibodies 11-fibrau (1:100 for 60 minutes) (clone D7-fib, Imgen, distributed by ITK Diagnostics, Uithoorn, The Netherlands), mouse anti-human CD68 (1:100 for 60 minutes) (M0814, DAKO, Heverlee, Belgium) or control mouse IgG2aκ (X0943, DAKO) and IgG1κ (X0931, DAKO) respectively. Endogenous Peroxidase activity was blocked with 3% H_2O_2 (Merck Millipore, Amsterdam, The Netherlands) in methanol. Subsequently, the sections were incubated with the secondary HRP-conjugated rabbit-anti-mouse IgA/G/M (1:200 for 60 minutes) (P0260, DAKO). Peroxidase was

developed with diaminobenzidine and counterstained with hematoxylin for 60 seconds.

Plasmid cloning and lentivirus production

For the production of lentiviral vectors, we made use of the third generation self-inactivating lentiviral (sin) vector system. The vector for pRRL-cPPT-CXCL10p-IL10-PRE-SIN has been described previously.[12] To obtain the pRRL-cPPT-PGK-IL10-PRE-SIN vector, the PGK-luciferase vector from our previous studies[18] was predigested and the CXCL10p-IL10 vector was digested with SalI and NheI (New England Biolabs, Ipswich, MA, USA) and the IL-10 gene was ligated in the predigested PGK promoter vector. For generation of the CXCL10p-fluc-RPL22p-rluc dual luciferase vector, a new multiple cloning site (mcs) was inserted in the SIN vector between the SalI and NheI sites, containing restriction sequences for XhoI-AgeI-SpeI-PmeI-AfeI-AscI-SalI-HpaI-NheI. The XhoI overhang from the mcs was compatible with the SalI overhang from the SIN vector. The mcs was used to clone the *RPL22* promoter (cloned from human gDNA (Promega), the renilla luciferase gene from the pMCS-green Renilla luciferase vector (ThermoScientific, Waltham, MA, USA) and the *CXCL10* promoter in one construct. The promoter from the *RPL22* gene was selected based on low variability in multiple microarrays.[19] All primer sequences are listed in Table 1. The lentivirus production, purification and quantification were performed as described previously [20]. Transduction of micromasses was performed with 150 ng virus/micromass in complete RPMI medium supplemented with 8 µg/ml polybrene (Sigma).

Confocal microscopy

For confocal microscopy, day 9 micromasses produced from an OA patient were transduced with lentiviral PGK-GFP and fixed in 1% paraformaldehyde, 48h after transduction. Micromasses were stained with DAPI (Molecular Probes, Oregon, USA) at a concentration of 0.2 ug/ml in PBS for 10 minutes, followed by three washing steps in PBS. Micromasses were mounted in Fluoromount-G (Southern Biotech, Alabama, USA). Confocal pictures were taken at 200x magnification using the Olympus Fluoview FV1000 laser scanning microscope (Olympus, Zoeterwoude, The Netherlands). DAPI and GFP were imaged using lasers at excitation wavelengths of 405 nm and 488 nm respectively. Image processing was performed using the Fluoview Viewer Sofware V4.1 (Olympus).

Flow cytometry

For flow cytometry analysis, micromasses were melted on ice for 2h, 48h after

transduction with lentiviral PGK-GFP. The cell suspension was incubated with antibodies 11-Fibrau and subsequently with donkey-anti-mouse conjugated to Alexa Fluor 568 (1:100 for 30 minutes) (A-10037, Thermo Scientific), or cell suspensions were incubated with mouse-anti-human CD68, conjugated to PE (1:20 for 60 minutes) (12-0689, eBioscience, San Diego, CA, USA). The flow cytometry was performed on the FACS Cyan (Beckman Coulter, Woerden, The Netherlands) using the 488 nm laser at the FL1, FL3 and FL7 channels for GFP, 11-Fibrau and CD68 respectively.

Luciferase measurements

The luciferase measurements were performed in 96-well white clear-bottom plates (Greiner Bio-one) using the Dual-Glo luciferase assay system (Promega). Micromasses were transferred to a well and lyzed in Dual-Glo reagent for 10 minutes. Firefly luciferase light production was measured on a Clariostar (BMG, Offenburg, Germany). Subsequently, the lysates were incubated for 10 minutes with Stop & Glo before measuring the Renilla luciferase light production. The values were corrected for the background signal and depicted as relative light units (RLU).

RNA isolation and qPCR

RNA isolation and qPCR were performed as described previously.[20] The primer sequences are listed in **Table 1**.

Table 1: List of oligonucleotide primer sequence

Oligo description	Sequence (5'→3')
MCS_oligoA	TCGAGACCGGTACTAGTGTTTAAACAGCGCTGGCGCGCCGTCG ACGTTAACG
MCS_oligoB	CTAGCGTTAACGTCGACGGCGCGCCAGCGCTGTTTAAACACTA GTACCGGTC
RPL22_prom_FW	TTTTACTAGTGGCGGCCTGGCTACAGCAAA
RPL22_prom_RV	TTTTGGATCCGGCGGCAGCGGAGTTAGAAAG
GAPDH_qPCR_FW	ATCTTCTTTTGCGTCGCCAG
GAPDH_qPCR_RV	TTCCCCATGGTGTCTGAGC
SOCS3_qPCR_FW	TCGGACCAGCGCCACTT
SOCS3_qPCR_RV	CACTGGATGCGCAGGTTCT
TNFa_qPCR_FW	TCTTCTCGAACCCCGAGTGA
TNFa_qPCR_RV	CCTCTGATGGCACCACCAG
IL-1b_qPCR_FW	TGGGTAATTTTTGGGATCTACACTCT
IL-1b_qPCR_RV	AATCTGTACCTGTCCTGCGTGTT

Multiplex ELISA assay

Cytokine concentrations were determined by luminex multianalyte technology on the Bio-Plex 200 (Bio-Rad, Hercules, CA, USA) in combination with Bio-Plex pro human cytokine kits (Bio-Rad) according to the manufacturer's protocol. For IL-10 and IL-6 measurements, the micromass culture supernatants were first diluted 25x. Samples below the detection limit were set at the lowest measureable quantity to perform statistical analysis.

Statistical analysis

Statistical analysis was performed using the Student's t-test, 1-way ANOVA and 2-way ANOVA. Results are depicted as mean +/- SD and P-values < 0.05 were regarded as significant. For statistical comparisons between conditions including multiple patients, patients were first individually normalized for the control conditions.

Results

The synovial micromass membrane contains FLS and MLS.

In previous studies that used the synovial micromass model, primary FLS were used that had been cultured for multiple passages. However, sustained culture of FLS can result in phenotype alterations[21] and in FLS micromasses the contribution of MLS is omitted. Therefore, we first tested the ability of synovial cell suspensions derived from digested synovium to form a lining layer in micromass culture. After 7 days, cells had migrated to the micromass-medium interface and resembled a synovial membrane **(Fig. 1)**. The sections were stained using the 11-fibrau antibody, which revealed that most cells in the lining are synovial fibroblast-like cells **(Fig. 1B)**. In addition, we performed a staining for the macrophage marker CD68. Macrophages were also present in the micromasses and appeared in the lining at day 7 **(Fig. 1D)**. This shows that the synovial micromass model can be used to study a membrane that resembles the architecture and composition of the synovial lining. When the micromasses were transduced with a lentiviral PGK-GFP vector, GFP expression was mainly observed in the lining layer **(Fig. 1E)** and to a lesser extent in the sublining.

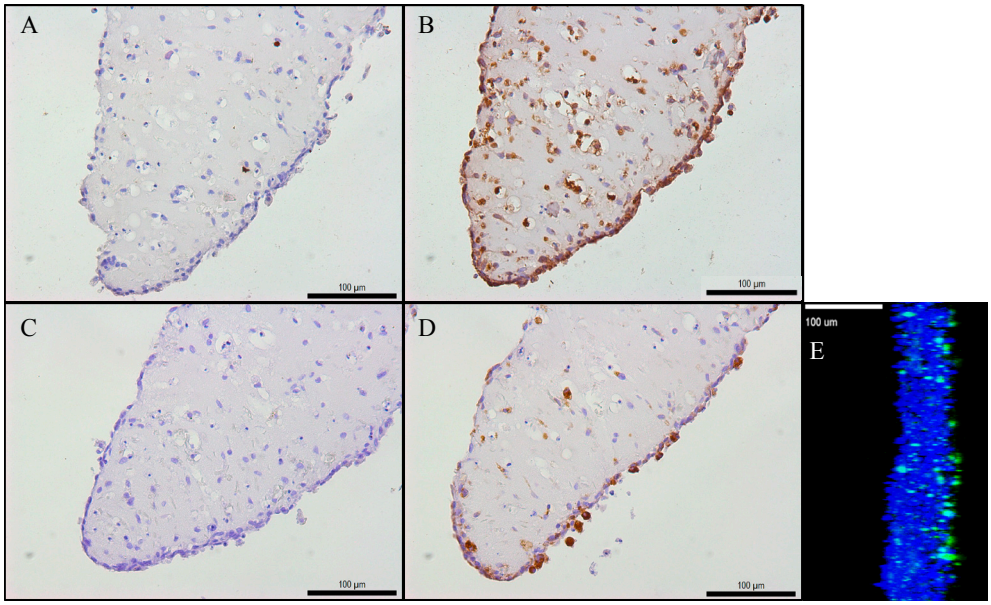


Figure 1: Immunohistochemical detection of fibroblasts and macrophages in synovial micromasses. Synovial micromasses were generated from digested synovial tissue cell suspension and cultured for 7 days. **(A)** IgG control antibody for 11-Fibrau staining. **(B)** 11-fibrau (brown) staining for synovial fibroblasts. **(C)** IgG control antibody for CD68-staining. **(D)** CD68-staining (brown) for macrophages. **(E)** Confocal fluorescent image of the micromass after transduction with lentiviral PGK-GFP. Side view with DAPI (blue) and GFP (green) staining. The micromasses used in Figure 1A-D were derived from RA material and similar results were obtained staining OA micromass sections.

The transduction efficiency and virus tropism were assessed using flow cytometry. Around 6% of the cells expressed GFP after transduction and both FLS (6.7% of the 78.7% 11-Fibrau-positive cells) and MLS (5.3% of the 38.0% CD68-positive cells) were transduced **(Fig. S2)**.

The *CXCL10* promoter responsiveness in synovial micromasses

The micromasses were transduced with a lentiviral vector to study the response of the*CXCL10* promoter, the promoter of our choice to obtain autoregulated transgene expression. The vector contained a CXCL10p-firefly luciferase reporter and an RPL22p-renilla luciferase gene to correct for micromass cellularity and transduction efficiency. Micromasses were transduced after forming a lining to resemble the *in vivo* synovial membrane transduction and stimulated with LPS, TNFα or IL-1β for 6 hours. Previous studies have found an upregulation of multiple plasma proteins

in the OA synovial fluid, which similar to DAMPs can activate macrophages in a TLR-4-dependent manner.[13] We therefore included the TLR-4-specific stimulus LPS in our studies. Although not significant for every individual patient, stimulation with LPS, TNFα or IL-1β resulted in a significant upregulation of *CXCL10* promoter activity **(Fig. 2A-C)**. These results show that the cells in the micromass lining can be transduced by lentiviruses and disease-related triggers can activate the *CXCL10* promoter. Interestingly, one patient (OA4) showed no significant response to any of the stimuli.

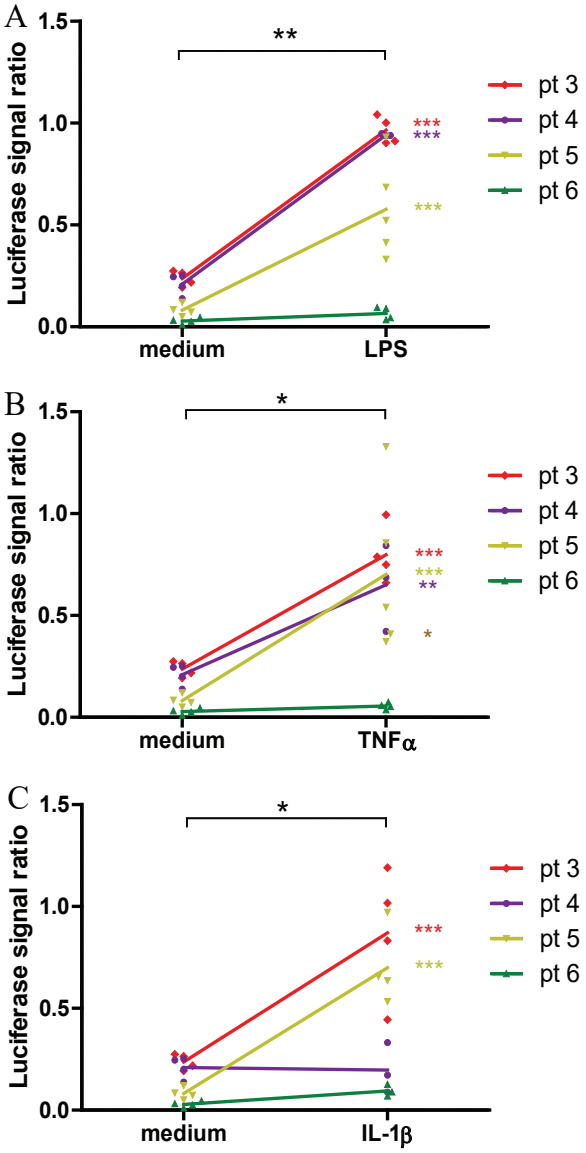
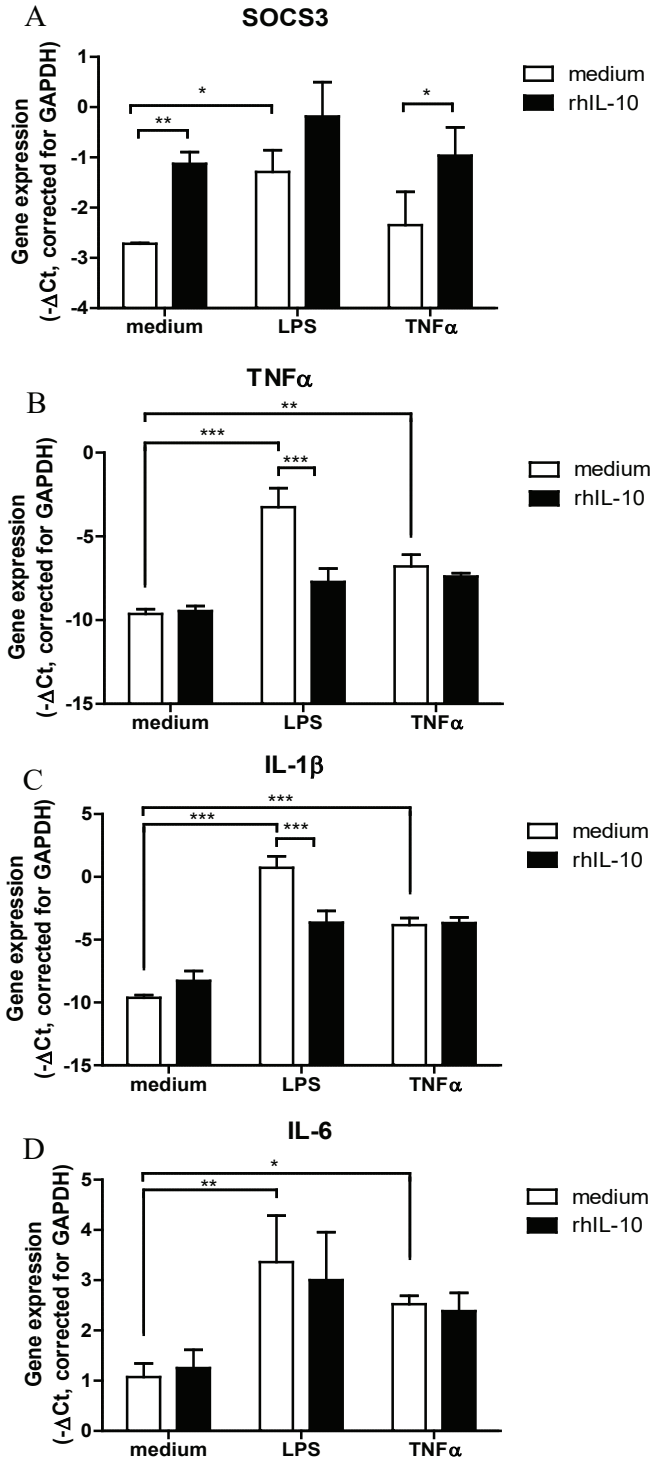


Figure 2: Activation of the *CXCL10* promoter in synovial cell micromasses by LPS, TNFα and IL-1β. Synovial micromasses of 4 OA patients were transduced with the CXCL10p-fluc-RPL22p-rluc dual luciferase construct and stimulated for 6h with **(A)** 100 ng/ml LPS, **(B)** 10 ng/ml TNFα or **(C)** 10 ng/ml IL-1β and compared to the unstimulated medium condition. The signal ratio was calculated as fireflyRLU/renillaRLU +/- SD. Micromasses are depicted as individual points and different colours represent different patients. Statistical analysis between medium and stimulated condition was performed by Student's t-test and comparisons within individual patient samples were calculated by 2-way ANOVA. * =P<0.05, ** =P<0.01.

The effects of IL-10 protein on synovial micromasses

After having established that the *CXCL10* promoter can mediate regulated expression in OA synovial micromasses, we set out to determine whether the transgene IL-10 can be a potential candidate for local treatment of OA. We first determined if IL-10 can lead to SOCS3 expression in the synovial micromasses. After 2h stimulation with recombinant IL-10, SOCS3 was significantly upregulated **(Fig. 3A)**. In addition, SOCS3 expression could also be induced by LPS and TNFα as previously described.[22] Stimulation of the micromasses with LPS and TNFα for 4h resulted in significant upregulation of TNFα and IL-1β mRNA **(Fig. 3B,C)**. In LPS-stimulated conditions, IL-10 could reduce this induction. IL-6 was also upregulated after pro-inflammatory stimulation **(Fig. 3C)**, but IL-10 could not reduce IL-6 expression. These results show that cells from the synovial micromass are responsive to IL-10 and downregulate the production of cytokines after stimulation, possibly via the induction of SOCS3.


A
SOCS3
Gene expression
(-ΔCt, corrected for GAPDH)
1
0
-1
-2
-3
-4
*
**
*
medium
LPS
TNFα
medium
rhIL-10
B
TNFα
Gene expression
(-ΔCt, corrected for GAPDH)
0
-5
-10
-15
**
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medium
LPS
TNFα
medium
rhIL-10
C
IL-1β
Gene expression
(-ΔCt, corrected for GAPDH)
5
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-5
-10
-15
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LPS
TNFα
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rhIL-10
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Gene expression
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5
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medium
LPS
TNFα
medium
rhIL-10

Figure 3: Gene expression in synovial micromasses after stimulation with LPS and TNFα in the absence or presence of IL-10.

Micromasses from synovial cell suspensions (3 per group) were cultured until lining formation was evident. Subsequently, the micromasses were stimulated for 2h or 4h with medium containing 100 ng/ml LPS, 10 ng/ml TNFα, 10 ng/ml IL-1β in the absence and presence of 10 ng/ml IL-10. Gene expression levels of SOCS3 at 2h **(A)**, TNFα at 4h **(B)**, IL-1β at 4h **(C)** and IL-6 at 4h were measured. Expression levels are depicted as threshold cycle (Ct) +/- SD, corrected for GAPDH expression. Statistical comparison within stimulation groups was performed by 2-way ANOVA and between groups by 1-way ANOVA. * =P<0.05, ** =P<0.01, *** =P<0.001.

We subsequently determined if the inducibility of the *CXCL10* promoter can be used to provide relevant IL-10 levels in synovial micromasses. After formation of the lining, the micromasses were transduced with IL-10 lentiviruses under control of the constitutive active *PGK* promoter or the inducible *CXCL10* promoter. The IL-10 levels in the supernatant of the micromasses were determined after 24h stimulation with LPS and TNFα. Micromasses transduced with PGK-luciferase control virus only produced low quantities of IL-10, indicating that the background production of endogenous IL-10 is low, even after stimulation with LPS or TNFα **(Fig. 4)**. Under control of the *PGK* promoter, high levels of IL-10 were secreted into the supernatant, which did not significantly differ after stimulation with LPS or TNFα. In contrast, micromasses transduced with CXCL10p-IL10 showed an increase in IL-10 production after stimulation with either LPS or TNFα.

Next, we used the micromass culture supernatants to investigate if the IL-10 production from the transduced micromasses can influence the inflammatory response in the synovial lining. Under control conditions without stimulation, the micromasses already produced the pro-inflammatory cytokines IL-1β and IL-6 **(Fig. 5A,B)**. The production increased after stimulation with LPS or TNFα, which has also been observed *in vivo*.[23] The stimulatory effects of LPS and TNFα on the release of IL-1β could be abolished by both constitutive (84.8% after LPS and 83.9% after TNFα) and inducible (70.7% after LPS and 87.4% after TNFα) expression of IL-10. Similar effects were observed for LPS-induced IL-6 secretion **(Fig. 5B)**. After stimulation with LPS, the IL-6 production was decreased by 67.1% by PGK-IL10 and 71.0% by CXCL10p-IL10. TNFα was below detection limits in multiple unstimulated micromasses **(Fig. 5C)**. After stimulation with LPS, TNFα was produced by the micromasses, but virus treatment showed no significant effects. These results show

that treatment of a synovial lining with the inducible *CXCL10* promoter for the expression of IL-10 can reduce the production of pro-inflammatory cytokines and can inhibit stimulation of the lining.

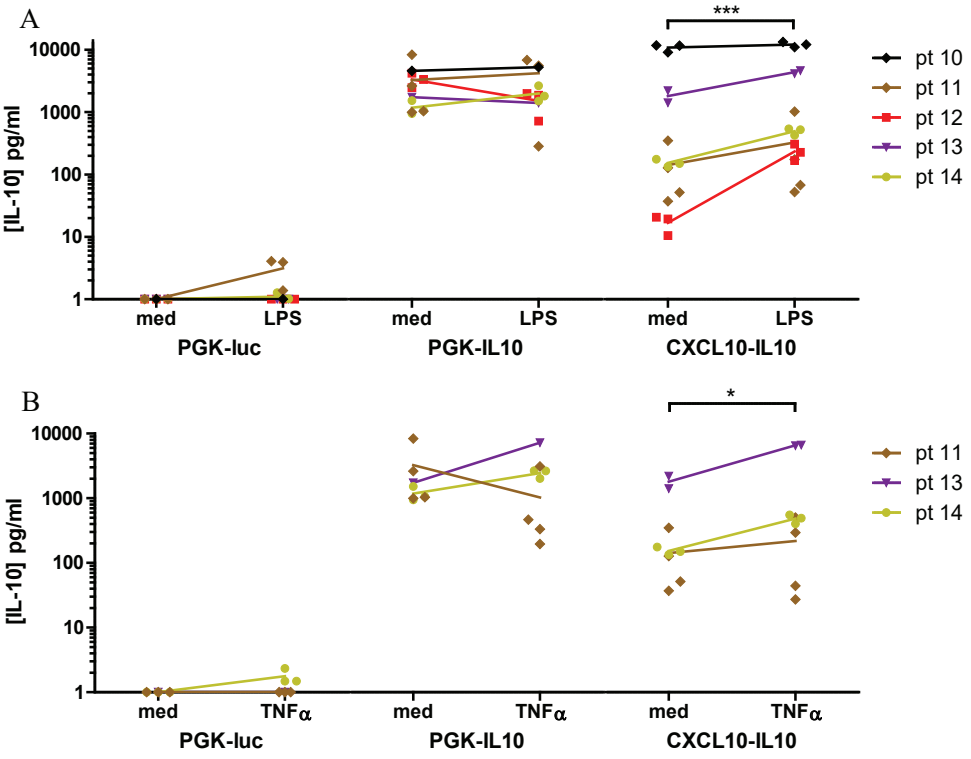

Figure 4: Production of IL-10 by transduced micromasses. Micromasses from synovial cell suspensions were transduced with lentiviral vectors coding for PGK-luciferase, PGK-IL10 or CXCL10p-IL10 after formation of a synovial lining. Subsequently, the micromasses were stimulated with medium containing 100 ng/ml LPS **(A)** or 10 ng/ml TNFα **(B)**. The IL-10 concentration in the supernatant after 24h was measured using a multiplex Elisa assay. Concentrations below 1 pg/ml were included in the statistical analysis, but are shown as 1 pg/ml in the Figure. An nsufficient number of micromasses could be generated from patients 10 and 12 to determine the response to TNFα. The medium, LPS- and TNFα-stimulated groups were compared by 1-way ANOVA. * =P<0.05, ** =P<0.01.

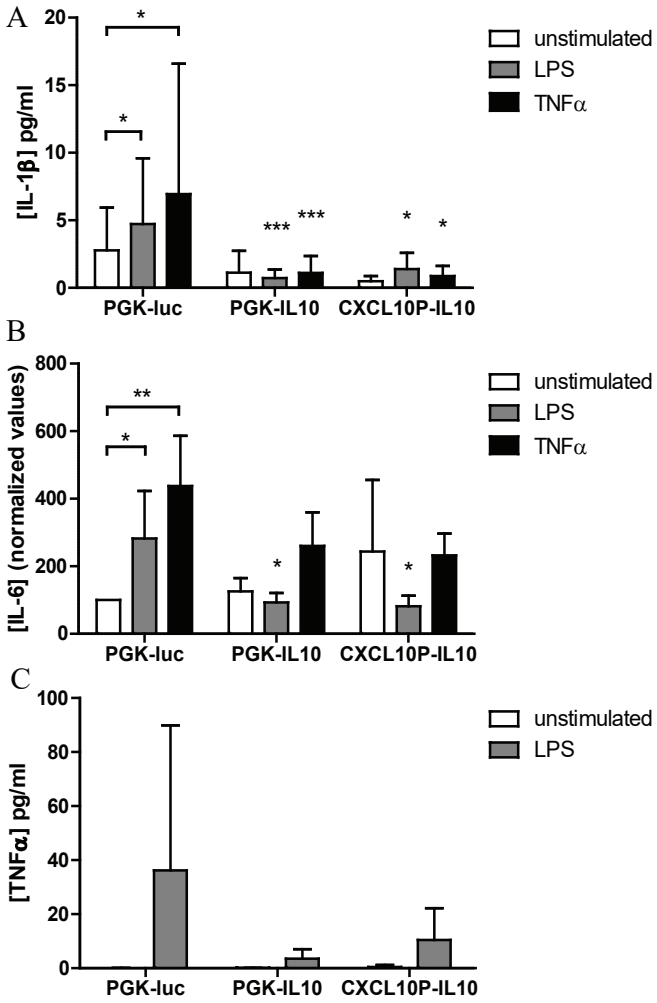

Figure 5: Cytokine production by stimulated micromasses treated with IL-10 viral vectors. Micromasses from synovial cell suspensions of 5 patients were transduced with lentiviral vectors coding for PGK-luciferase, PGK-IL10 or CXCL10p-IL10 after formation of a synovial lining. Subsequently, the micromasses were stimulated with medium containing 100 ng/ml LPS (all patients) or 10 ng/ml TNFα (3 patients). The concentrations of IL-1β **(A)**, IL-6 **(B)** and TNFα **(C)** in the supernatant after 24h were measured using a multiplex ELISA assay. IL-1β and TNFα could only be quantified in 3 patients. Because of high variations in IL-6 production between patients, the values were first normalized for every individual patient for PGK-luc unstimulated. The basal values (pg/ml) were $2.7x10^5$, $6.5x10^5$, $2.7x10^4$, $5.1x10^4$ and $3.3x10^5$ respectively. The medium, LPS- and TNFα-stimulated groups were compared by t-test. Significancies without a capped line were compared to PGK-luc. * =P<0.05, ** =P<0.01, *** =P<0.001.

Discussion

In this study we set out to study the therapeutic potential of disease-inducible gene therapy for OA using the CXCL10p-IL10 lentivirus in a synovial lining model resembling early stage OA that includes both synovial fibroblasts and synovial macrophages. After digestion of a synovial tissue sample the cells were mixed with Matrigel to obtain a synovial micromass, in which both FLS and MLS migrated to the micromass edge to form a lining. After transduction with a lentiviral CXCL10-promoter reporter vector, the *CXCL10* promoter could be significantly induced by pro-inflammatory stimulation. When the *CXCL10* promoter drived the expression of the gene coding for anti-inflammatory IL-10, sufficient IL-10 could be produced to reduce the release of IL-1β and IL-6 after stimulation with TNFα or LPS.

In vitro studies with human synovial cells are often performed in monolayer culture, in which the cells might behave and interact differently from the complex structural organization in the joint.[24] To improve *in vitro* synovium studies, Kiener at al. developed a 3D synovial lining model.[25] Primary FLS are mixed with matrigel to form a micromass structure, in which the FLS migrate towards the outside to form a lining layer, a phenomenon not observed with dermal fibroblasts. The micromass lining shows many similarities to the synovial lining. It expresses the synovial fibroblast marker PRG4 and produces extracellular matrix.[26] MLS were not included in these studies, but the FLS could support the survival of monocytes from peripheral blood.

Interestingly, macrophages in culture without the appropriate stimulation undergo apoptosis.[27] Kiener et al. showed that primary monocytes from the blood are not viable in micromasses when cultured alone, but could survive for over 3 weeks when cultured together with FLS.[26] We showed that the micromasses can also support the survival of synovial macrophages and that the macrophages resided in the lining. Because the micromasses do not show the fibrotic changes and macromolecular cartilage and bone detritus-rich synoviopathy observed in late stage OA, the synovial micromass might more closely mimic early stage OA compared to synovial explants from joint replacement remnants. This enables the possibility to test therapeutic strategies in an early disease stage where inhibition of inflammation might prevent disease progression.

During inflammation, synovial macrophages become activated and produce pro-inflammatory cytokines, matrix-degrading enzymes and promote synovial hyperplasia.[28] The synovial micromass model based on primary tissue digestions can be a suitable model to study these processes. The inclusion of MLS in the micromasses

is particularly important for testing IL-10-based therapies, since the primary receptor subunit for IL-10, IL-10R1, is most strongly expressed on cells of hematopoietic origin.[29,30] After transduction of the micromasses with lentiviral PGK-GFP, transgene expression was predominantly observed in the membrane, which is similar to observations with intra-articular injections of lentivirus *in vivo*,[31] providing further support that micromasses are an adquate model for the synovial membrane.

IL-10 is a powerful anti-inflammatory cytokine and IL-10-based gene therapy has proven to be effective in multiple animal models of OA and cartilage damage, alone and in combination with IL-1 receptor antagonist (IL-1Ra) or IL-4.[32,33] Low innate production of IL-10 by blood cells upon lipopolysaccharide (LPS) stimulation *ex vivo* is associated with an increased risk of OA.[34] In addition, it has been concluded from a systematic review that physical exercise has positive effects on pain and disability in knee OA.[35] These effects might be mediated by IL-10, which was found to be upregulated in the synovial fluid after exercise.[36] In addition to the inhibition of inflammation and matrix-degrading enzymes, IL-10 has chondroprotective and anabolic effects on cartilage by stimulating chondrocyte proliferation, stimulating the production of extracellular matrix components and reducing chondrocyte apoptosis.[2,37] One of the mechanisms by which IL-10 can exert its anti-inflammatory effects, is by inducing the expression of SOCS3.[38] SOCS3 can inhibit JAK-STAT signaling induced by cytokine receptor activation.[39] A second anti-inflammatory mechanism proposed for IL-10 is the inhibition of proteins that stabilize TNFα mRNA.[40] As a result, the TNFα mRNA becomes prone to degradation mediated by the 3'UTR AU-rich elements (AREs). We have found both upregulation of SOCS3 and reduced levels of TNFα mRNA after treatment with recombinant IL-10, indicating that IL-10 might inhibit the inflammatory response in synovial micromasses at multiple levels.

The levels of TNFα, IL-1β and IL-6 are increased in OA patients and high levels are associated with increased radiographic progression.[41] We have observed increased production of these cytokines by the micromasses under inflammatory conditions. TNFα and IL-1β mRNA could be down-regulated by IL-10 and the produced levels of IL-1β and IL-6 decreased after treatment with both PGK-IL10 and CXCL10p-IL10. Interestingly, the variation in basal IL-10 production between patients after transduction with CXCL10p-IL10 was bigger compared to the IL-10 production after stimulation with LPS or after transduction with PGK-IL10 **(Fig. 4)**. The variation in basal IL-10 production from the CXCL10p-IL10-transduced micromasses might result from a variation in tissue composition or differences in inflammatory imprinting of synovial cells between patients rather than transduction efficiency, because less variation in IL-10 production after transduction with PGK-

IL10 was observed.

Conclusions

We showed that 3D micromasses that are made from primary synovial cells of OA patients form a synovial lining consisting of fibroblast-like and macrophage-like synoviocytes and the micromassas might be a useful tool to study synovium *in vitro*. The micromass lining can be transduced by lentiviruses and provide disease-inducible expression of sufficient amounts of IL-10 to reduce the production of pro-inflammatory cytokines by the micromasses. Gene therapy with the CXCL10p-IL-10 vector might be a promising strategy for local treatment of early OA.

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We would like to thank Jon San Liu for his CD68 staining of the synovial paraffine sections.

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Ethics approval and consent to participate

The patient samples used in this study were obtained after surgical intervention and therefore not part of the Medical Research Involving Human Subjects Act (WMO). Instead, we adhered to the Human Tissue and Medical Research: Code of Conduct for Responsible Use (2011) of the Stichting Federatie Medisch Wetenschappelijke Verenigingen (FEDERA) and the protocols were approved by the local CMO-light committee. All patients gave their informed consent.

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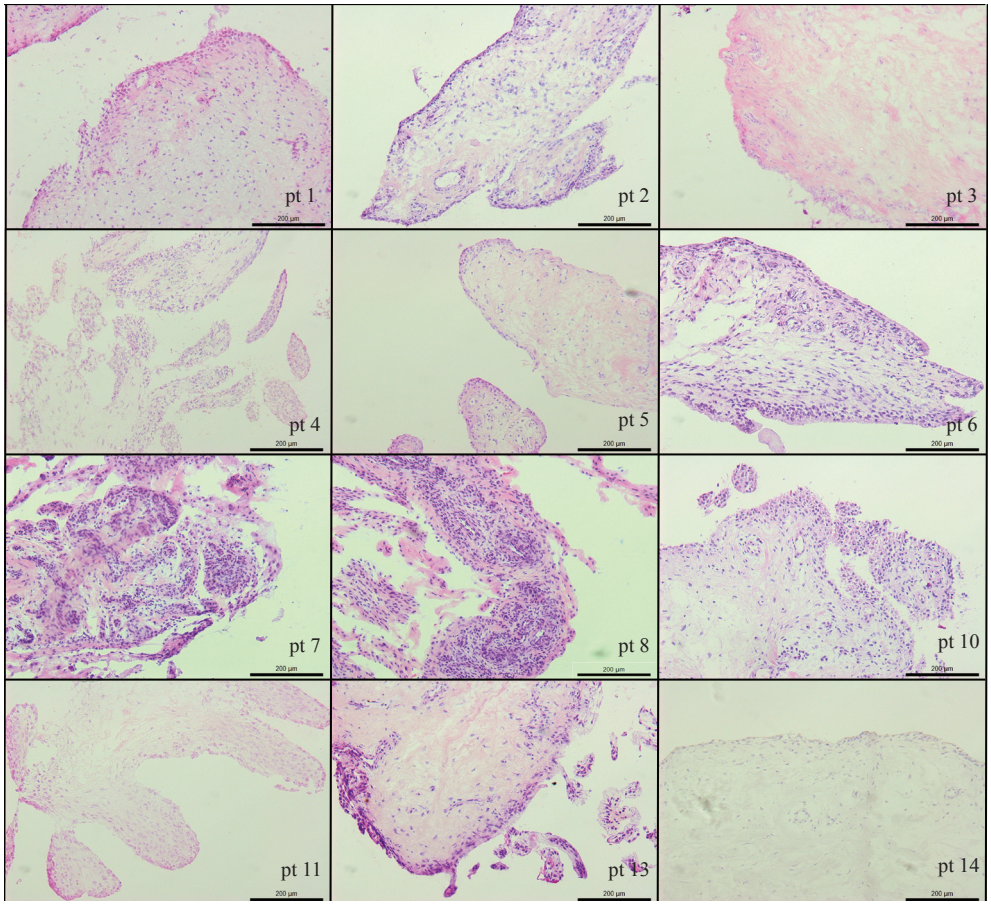
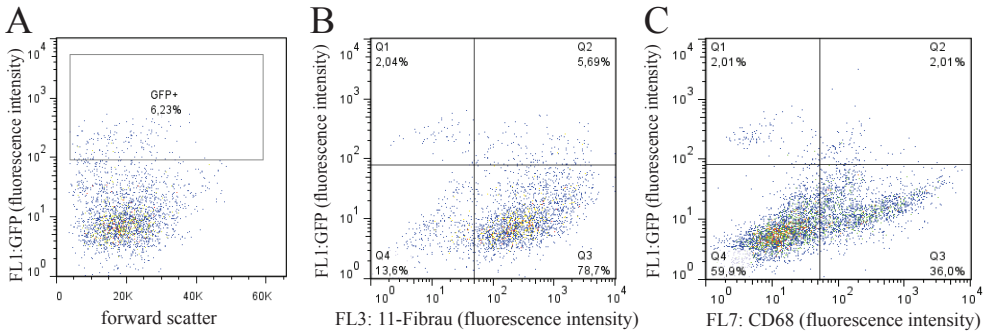
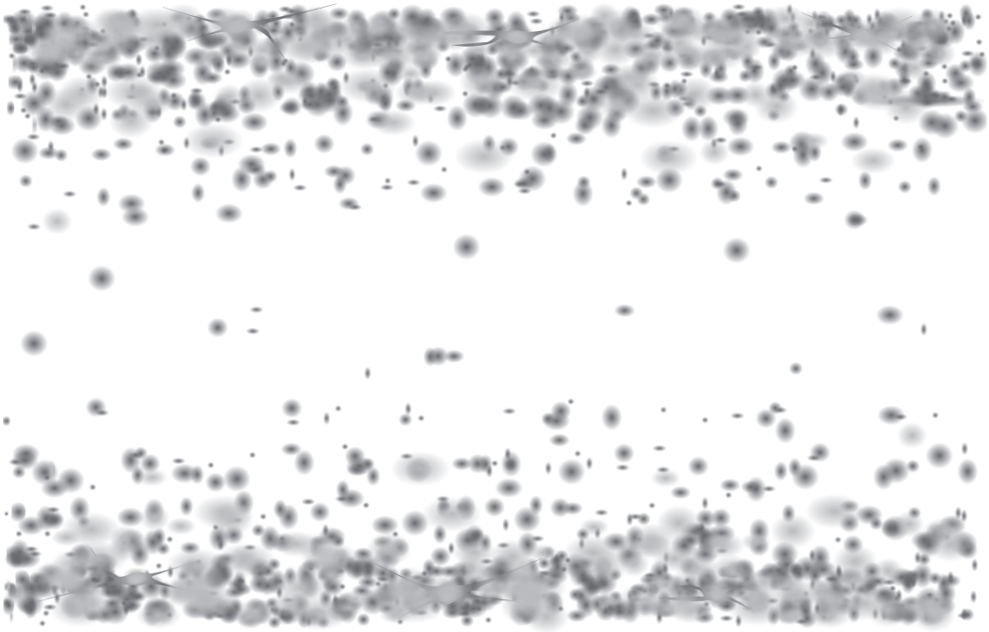
Supplementary Figures



Supplementary Figure S1: HE stainings of 7 µm cryosections obtained from patient biopsies used in this study.



Supplementary Figure S2: Scatter plots of flow cytometry analysis of micromasses transduced with PGK-GFP. 7 days after formation, the micromasses were transduced with lentiviral PGK-GFP. 48h after transduction, the micromasses were melted and stained for FLS (11-Fibrau) and MLS (CD68) markers. Cells were first gated for the live gate. **(A)** GFP signal and forward scatter **(B)** GFP signal and 11-Fibrau signal **(C)** GFP signal and CD68 signal. The scatter plots are representative for multiple experiments.



Chapter 8

Summary and final considerations

Gene therapy in murine models of arthritis

Rheumatoid Arthritis (RA) is a systemic autoimmune disease affecting joints of an estimated 1% of the world population. Despite much research effort in the identification of risk factors and the underlying immunology, many questions still remain about the disease etiology. Although RA still cannot be cured most patients can nowadays be successfully treated using the available medication, including the disease-modifying drugs (DMARDs) methotrexate and biological drugs. These drugs are injected on a regular basis and cause continuous systemic immunosuppression that can lead to side effects like opportunistic infections. The fact that the biologicals are protein-based drugs sparked the hypothesis that the medication might also be produced by cells in the synovium. By transducing the synovial cells with a viral vector encoding a therapeutic protein, the inflammation can be locally inhibited. An additional level of regulation that can be introduced using this approach is by using inflammation-sensitive promoters that induce the transcription of the drug only during active disease. The local and disease-responsive production of biologicals could be a long-term solution for affected patient joints, while minimizing systemic side effects.

We set out to explore this approach in a murine model of RA in **Chapter 2**. In microarray data of streptococcal cell wall (SCW)-induced arthritis, several genes were found to be upregulated compared to healthy joints. The promoters of these genes were possible candidates for regulated gene expression and cloned in front of a luciferase reporter gene. These promoter-reporter constructs were injected in mouse joints and the light production by the luciferase reporter was assessed during SCW-induced arthritis. The *Saa3* and *Mmp13* promoters performed best with a high activity in diseased joints compared to healthy joints and activation peaks at day 1 and day 4/7 respectively. For therapeutic application, the luciferase reporter gene was replaced by the anti-inflammatory Interleukin-10 (IL-10). In SCW-induced arthritis, the Saa3p-IL10 and Mmp13p-IL10 significantly reduced synovitis and proteoglycan depletion in the murine knee joint and the systemic Keratinocyte Chemoattractant (KC) levels. These therapeutic effects were associated with increased expression of Suppressor of Cytokine Signaling 3 (SOCS3) and IL-1 Receptor Antagonist (IL-1RA) in the synovial tissue. Ideally, future development of RA drugs based on this approach can result in a therapy for the treatment of joints refractory for current medication strategies with low off-target exposure.

A more challenging joint disease to obtain clinical remission is osteoarthritis (OA). Although OA is the most common joint disease, no drugs are available to modify the disease course. Despite the probable involvement of wear-and-tear,

OA has many pathological changes in common with RA on a local level, including the influx of inflammatory immune cells, the production and activation of matrix-degrading enzymes and synovial fibrosis. Many proteins have therefore been suggested to have therapeutic potential, but systemically injected proteins hardly reach the joint and proteins injected in the joint are rapidly cleared. We therefore hypothesized that gene therapy could be a promising strategy to test the potential of protein-based drugs for the treatment of OA. In **Chapter 3**, Tumor Necrosis Factor Alpha (TNFα)-stimulated gene 6 (TSG-6) was selected to inhibit OA, because of the anti-inflammatory properties, protection against cartilage damage in experimental RA, inhibition of Metalloproteinase (MMP) activation and inhibition of bone remodeling.[1-3] *In vitro* constitutive overexpression of TSG-6 in osteoclasts could reduce the bone resorption of dentin slices, showing the biological activity of our viral vector. *In vivo*, we tested the TSG-6 lentiviral vector in the Collagenase-induced arthritis model, which features inflammation at early time points and results in joint damage at day 42. However, in the TSG-6 lentivirus-treated group we observed no reduction in inflammation and no reduction of cartilage damage. Instead, ectopic bone formation occurred at the medial collateral ligament, which could be observed on histology and X-ray analysis. The ectopic bone formation might be the result of a disturbed repair mechanism and we believe that TSG-6 is not suitable for OA gene therapy. The search for disease-modifying OA drugs will therefore continue and ideally, a therapy should first prove successful in murine models of OA before proceeding to living humans.

Testing potential therapies in a human setting: tackling the challenges

The translation of the findings in mouse models to the human system commonly starts with *in vitro* assays before proceeding to clinical trials. Many *in vitro* experiments rely on primary biopsies with a disease phenotype obtained from patients. Several practical and scientific challenges are encountered when designing these experiments. For example, primary material should be used as soon as possible before the disease phenotype of the tissue is lost. Because the patient donors required for the studies are treated one-by-one at irregular times at different days, this poses a logistic challenge. To enable researchers to synchronize the use of biopsies from several donors, we have studied the effects of several cryopreservation media on synovial biopsies in **Chapter 4**. The goal of this study was to find a slow-freezing cryopreservation protocol that did not influence the cell viability, histological integrity of the tissue, disease phenotype of the cells and inflammatory response of

the biopsy. From several commercially available cryopreservation media, we found that CS10 and CryoSFM could both provide storage of the biopsies in liquid nitrogen without loss of the investigated variables. The establishment of an easy-to-perform and robust cryopreservation protocol is essential for the development of a synovial tissue biobank with live patient biopsies.

In vitro experiments with biopsies have an added value compared to monolayer culture, because the biopsies contain all cell types found in the diseased synovium and the cells maintain their important cell-cell and cell-matrix interactions. A disadvantage of the biopsies is that many disease processes, like hyperplasia and fibrosis have already occurred. In addition, a large variability between multiple biopsies from within a single patient can be observed. To improve the 3-dimensional culture options for human synovium, we further investigated the synovial membrane model initially developed Kiener et al.[4] In these experiments synovial fibroblasts were mixed with cold liquid Matrigel. At 37°C the Matrigel became solid and the cells migrated toward the surface and formed a lining layer similar to the lining layer in synovium. In **Chapter 5** we analyzed the composition and behavior of cells in micromasses constructed from complete synovial biopsy digestions. Synovial fibroblasts and synovial macrophages were the cell types that predominantly survived in the micromass and both cell types appeared in the lining after 7-14 days culture. Additional cell types from the synovial biopsies, including T cells, B cells and epithelial cells disappeared over time under our culture conditions. Lining hyperplasia could be observed after 3 weeks stimulation with TNFα, a pathological phenomenon also observed in inflamed synovium. The synovial micromasses could also be cryopreserved without loss of sensitivity to inflammatory stimuli. Synovial biopsies can thus be digested and the complete cell suspension can be used to generate micromasses when the material becomes available and cryopreserved after lining formation. Subsequently, at the start of an experiment the appropriate number of micromasses from the required amount of donors can be thawed.

CXCL10p-IL10 lentiviral gene therapy

The *Saa3* promoter, which showed the strongest inflammatory response in the murine arthritis, was not the most suitable for application in the human system. In humans, *Saa3* is a pseudogene not coding for a protein, so the human *Saa3* promoter sequence is therefore no promising candidate for regulation of transgene production. The promoter response of the murine *Saa3* promoter in human cells was inferior to other promoters (not shown). We therefore initiated a new search for disease-responsive promoters to provide disease-responsive IL-10 gene therapy

in human cells. In **Chapter 6**, we compared microarrays of synovial tissue of RA patients with healthy control synovium and selected all genes that were significantly upregulated at least 10-fold in RA compared to healthy controls with a minimum average probe signal intensity in RA of 500 (arbitrary units), which resulted in a list of 22 genes. In lipopolysaccharide (LPS)-stimulated synovial cell suspensions we determined which of these genes was most responsive to inflammatory stimulation in our target cell population. The *CXCL10* gene showed the highest induction and the *CXCL10* promoter was cloned in a luciferase reporter construct for further analysis. Using this reporter vector we found that the *CXCL10* promoter was activated by the serum of RA patients and showed a strong reaction to TNFα with a peak after 8-12 hours. The promoter activity decreased in time as the TNFα concentration dropped, but could be re-activated by TNFα after 96h. The regulated expression is pivotal for the offer-meets-demand behavior of the intended gene therapy. When the luciferase transgene was replaced by the human IL-10 gene and synovial cell suspensions were transduced using the CXCL10p-IL10 lentiviral vector, the cells could produce IL-10 which increased after stimulation with LPS. The produced IL-10 levels could adequately reduce the production of pro-inflammatory cytokines by the synovial cells, most notably TNFα and IL-1β.

We subsequently tested the CXCL10p-IL10 lentiviral vector in synovial micromasses produced from biopsies from OA donors to assess the anti-inflammatory potential of the gene therapy in an inflammatory OA setting in **Chapter 7**. Similar to the experiments using cell suspensions, we observed increased production of IL-10 by the micromasses after stimulation with LPS or TNFα. In CXCL10p-IL10-treated micromasses, we observed a decrease in production of pro-inflammatory IL-1β and IL-6 both at basal level and after stimulation. The anti-inflammatory potential of the *CXCL10* promoter-based IL-10 expression was as efficient as constitutive expression by the *PGK* promoter. Similar to the IL-10 overexpression in the mouse model in chapter 2, we observed increased expression of SOCS3 in IL-10-stimulated micromasses, which might be an important mediator of the anti-inflammatory IL-10 effects.

In conclusion, we have shown in this thesis the potential of disease-inducible IL-10 gene therapy for the treatment of inflammatory joint disease in murine arthritis and in a well-characterized 3D model of the human synovial lining.

Future considerations

Gene therapy for RA and OA

The history of gene therapy and the cornerstone clinical trials that lead to the 3 currently approved gene therapy drugs are described in the introduction. The initial focus of gene therapy research was on treating lethal genetic defects, but most current clinical trials are directed against different types of cancer. However, an increasing amount of trials is also addressing non-lethal diseases, such as retinal disorders, RA and OA. Although inducible gene therapy for RA has the potential to provide long-lasting treatment with minimal side effects, much revenue is currently generated with quite effective medication which reduces the appeal to invest in gene therapy by pharmaceutical companies.[5] Despite this hurdle, a phase I gene therapy clinical trial was approved in 2016 for the Dutch company Arthrogen to test an AAV5 vector with Interferon-β under control of an artificial promoter containing repeats of NFκB transcription factor binding sites. Interestingly, IL-10 has been identified as one of the predominant downstream proteins responsible for the anti-inflammatory effects of Interferon-β.[6,7] The results and safety analysis of this vector will be very important for future trials for RA gene therapy. In contrast to RA, there is no effective treatment that can slow down the progression of OA. With the increasing evidence that OA is not only wear-and-tear, it might be expected that more funds will be invested in the development of a treatment for OA, compared to RA. The added value of gene therapy in this field can be to provide long-term bioavailability of protein-based drugs in the joint. The primary emphasis will therefore be on finding proteins with therapeutic effects, rather than fine-tuning the expression of available transgenes with inducible promoters. In this thesis, we have found that in experimental OA, TSG-6 is not a suitable transgene, but in the micromass model IL-10 could inhibit the inflammatory response. The therapeutic effects of IL-10 can be further studied in models that not exclusively focus on inflammation.

In the initial phases of our research with lentiviral vectors, several reviews were published that advocated the use of AAV vectors for gene therapy instead of integrating viral vectors,[8,9] because of the low AAV pathogenicity and the potential danger of integrating viruses we contemplated whether to switch to an AAV vector. However, in recent clinical trials with modern integrating viruses that lack viral enhancers and the technical advances that enable single cell analysis, it has been

observed that with the latest generation of lentiviruses the integration sites do not correlate with subsequent cell proliferation.[10] The association between integration near pro-oncogenes and clonal expansion of blood cells had recently been observed in a clinical trial after retroviral transduction,[11] although it was later stated that no leukemia developed during 33 months follow-up of the 9 patients. Based on these observations we believe that a lentiviral vector-based IL-10 gene therapy can be a useful vector to provide long-term IL-10 expression. The increasing body of safety data from clinical trials with different vector types and the ever increasing follow-up time of patients from the recent trials will reveal the actual safety and long-term clinical efficacy of these vectors and eventually shape the public opinion on gene therapy for non-lethal diseases.

The *CXCL10* promoter

In our studies we put particular effort in the quest for the most optimal disease-inducible promoter. Instead of combining multiple repeats of transcription factor binding sites in a synthetic promoter, we looked for genes of which the promoter sequence has evolved to induce expression during disease. We therefore analyzed microarrays of synovial tissue and for human application found several genes that were highly increased in microarrays for RA tissue compared to healthy controls. To determine if the upregulation was not the result of a shift in cell composition of the biopsy, but rather an induction of expression, we checked the expression in stimulated synovial cells. The *CXCL10* promoter showed the highest induction and previous promoter mutation studies have shown that the *CXCL10* promoter contains functional binding sites for several different transcription factors and a TATA-box.[12,13] This indicates that the promoter can be activated by different triggers without the requirement of many *cis*-acting elements. However, an interesting improvement to the CXCL10 promoter could be obtained by including enhancer regions in the construct, similar to the previously applied IL1/IL6 promoter, which consisted of an IL-1 enhancer element coupled to the IL-6 promoter.[14] The ENCODE consortium has recently performed large-scale functional analysis of the human genome.[15] One of the key objectives was to map all distal enhancer elements to their associated promoters. One significantly correlated enhancer region was identified for the CXCL10 promoter, which was located ~466 kb upstream of the promoter region.[16] Although the enhancer has not been investigated, the incorporation of the enhancer in the lentiviral CXCL10-promoter constructs might increase the sensitivity to inflammatory stimuli or decrease the background activity of the promoter.

In addition to providing disease-responsive gene therapy, the inducible promoters might be used as reporter systems when coupled to the luciferase transgene. We have observed that the *Saa3* promoter is activated early during experimental arthritis and the *Mmp13* promoter activity peaked at a later time point, which indicates that the promoters correlate with acute inflammation and synovial repair mechanisms respectively. In addition, previous experiments have shown that the *Saa3* promoter is responsive to factors in the serum of RA patients[17] and we have observed a similar response with the *CXCL10* promoter. Because the promoter activation is the net result of activating and inhibiting factors, a combination of promoter reporters might reflect the disease activity and therapy response or be of prognostic value. There is great demand for accurate assays and the promoter reporters can easily be studied using the serum or synovial fluid of appropriate patient cohorts. In such assays, reporter cells stably transduced with promoter reporter constructs can be stimulated with patient serum and the amount of light production can reflect the disease activity in the patient. This might for example be used to determine the therapy response to expensive biologicals at early time points, so a switch to a different biological therapy can be made early on. For these experiments, we have several promoter-reporters available that were derived from the genes identified in the RA microarrays (Chapter 6, Table 1). Although we could not obtain sufficient transgene expression for gene therapy with several of these vectors (eg. promoters from *CXCL13*, *NMES1* and *CXCL9*), an application of these promoters in a reporter assay could still be interesting.

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Nederlandse samenvatting

Gentherapie in muismodellen van reumatische ziekten

Reumatoïde artritis (RA) is een chronische auto-immuunziekte die de gewrichten aantast van ongeveer 1% van de wereldbevolking. Ondanks de vele onderzoeken die zijn gedaan om de risicofactoren voor het ontwikkelen van RA te vinden, is nog steeds niet volledig bekend hoe de ziekte precies ontstaat. Het lukt op dit moment dan ook nog niet om RA te genezen en een moderne behandeling richt zich daarom op het onderdrukken van de ontstekingsreactie. Dit gebeurt onder andere met reumaremmers (disease-modifying anti-rheumatic drugs, DMARDs). Voorbeelden hiervan zijn methotrexaat en biologische reumaremmers op eiwit-basis (biologicals). Deze medicijnen worden vaak wekelijks geïnjecteerd en onderdrukken het immuunsysteem in het hele lichaam, waardoor bijwerkingen zoals infecties kunnen ontstaan. Omdat eiwitten door cellen gemaakt worden, is enige tijd geleden het idee geopperd om gewrichtscellen zelf de biologicals te laten produceren. Door met een virus het bouwplan (gen) voor een biological aan te bieden aan cellen in de binnenbekleding van het gewricht (synovium) kan de ontsteking mogelijk lokaal worden onderdrukt. Bovendien is het mogelijk een ziektegevoelige schakelaar in te bouwen in het bouwplan, waardoor de biological uitsluitend geproduceerd wordt tijdens perioden van actieve ontsteking. Door de lokale en ziektegevoelige productie van de medicijnen kan een gewricht met een enkele injectie mogelijk voor langere tijd worden beschermd, terwijl de bijwerkingen worden beperkt.

Dit concept zijn we eerst gaan verkennen in een muismodel voor RA in **Hoofdstuk 2**. We hebben gekeken in de ontstoken gewrichten van deze muizen welke genen hier vooral geactiveerd zijn in vergelijking met gezonde gewrichten. De schakelaars (promotors) van deze genen zijn mogelijke kandidaten voor de ziekte-gereguleerde therapie. Om deze promotors te onderzoeken, zijn ze gekopieerd uit het genoom en voor het gen van een lichtgevend luciferase-eiwit gezet. De hoeveelheid licht die hiermee wordt geproduceerd is een maat voor de activiteit van de promotor. Deze zogeheten promotor-reporters zijn geïnjecteerd in de gewrichten van muizen met experimentele artritis en vervolgens is gekeken naar de hoeveelheid licht in de gewrichten. De promotors die het meest geactiveerd werden door de artritis waren van de genen *Saa3* en *Mmp13*. Om de therapeutische mogelijkheden te bepalen met deze promotors, is het bouwplan voor de luciferase reporter vervangen door het ontstekingsremmende eiwit interleukine-10 (IL-10). Hierdoor ontstonden de virale medicijnen Saa3p-IL10 en Mmp13p-IL10. In de muizenknieën met experimentele artritis zorgden behandelingen met Saa3p-

IL10 en Mmp13p-IL10 voor een afname van ontsteking en voor verminderde effecten op het kraakbeen van het gewricht in vergelijking met muizen met een controlebehandeling. In het synovium zagen wij een verhoging van de productie van de ontstekingsremmende eiwitten Suppressor of cytokine signaling 3 (SOCS3) en IL-1 receptor antagonist (IL-1RA), wat mogelijk voor deze effecten heeft gezorgd. Daarnaast was er in het serum van de behandelde muizen een afname te zien van de ontstekingsmarker keratinocyte chemoattractant (KC). Deze benadering kan in de toekomst mogelijk leiden tot een behandeling voor gewrichten die nog niet op medicatie reageren met verminderde kans op bijwerkingen.

Een grotere uitdaging voor behandeling vormt de gewrichtsziekte artrose (osteoartritis, OA). Ondanks dat artrose de meest voorkomende gewrichtsaandoening is, zijn er nog geen medicijnen beschikbaar die aangrijpen op het ziekteproces. In klassieke opvattingen werd OA vaak uitsluitend gezien als gewrichtsslijtage, maar tegenwoordig zijn er veel aanwijzingen dat ook ontsteking een actieve rol speelt bij het ziekteproces. Dit zorgt voor enkele overeenkomsten met RA op lokaal niveau, zoals het binnendringen van ontstekingscellen, de productie van schadelijke enzymen en fibrose van het synovium. Verschillende eiwitten zijn in het verleden voorgesteld om OA te behandelen, maar intraveneus toegediende eiwitten bereiken het gewricht slecht en herhaalde injecties in het gewricht zijn onwenselijk. Wij verwachten daarom dat gentherapie een effectieve manier kan zijn om voor langere tijd eiwitten in het OA-gewricht geproduceerd te krijgen. Dit hebben wij onderzocht in **Hoofdstuk 3**. Het eiwit Tumor necrosis factor alpha (TNFα)-stimulated gene 6 (TSG-6) is geselecteerd voor de behandeling, omdat bekend is dat het in muismodellen van RA de ontsteking kan remmen, het kraakbeen kan beschermen en de productie van schadelijke enzymen kan verminderen. Helaas zagen wij van deze behandeling geen verbetering in de ontsteking of de kraakbeenschade. In plaats daarvan trad botvorming op in de gewrichtsbanden. Deze ongewenste bijwerking is mogelijk het gevolg van een verstoord reparatieproces. Op grond van dit resultaat bevelen wij TSG-6 niet aan als therapie voor OA. De uitdaging voor de behandeling van OA blijft voorlopig dus het vinden van een geschikt eiwit voor een gentherapie.

Het testen van mogelijke therapieën in mensen: de uitdagingen oplossen

Het vertalen van de bevindingen in muizen naar de menselijke situatie start vaak met reageerbuisproeven met menselijk materiaal voordat nieuwe medicijnen in de kliniek worden getest. Veel van deze experimenten zijn gebaseerd op biopten die uit patiënten worden gehaald en snel moeten worden gebruikt voordat dat het materiaal ziekte-eigenschappen verliest. Dit is uitdagend, omdat verschillende

patiënten die voor een studie nodig zijn op verschillen momenten behandeld worden. Om deze experimenten logistiek beter te kunnen stroomlijnen, hebben wij in **Hoofdstuk 4** gekeken naar een methode om de biopten levend in te vriezen. Het doel was om een invriesmethode te vinden die geen invloed had op de overleving, de vorm en de ontstekingsgevoeligheid van de biopten. Dit bleek te lukken als de biopten werden ingevroren in invriesvloeistof CS10 of CryoSFM. Het invriezen van biopten biedt de mogelijkheid om het materiaal van verschillende donoren tegelijk te gebruiken, maar biedt ook de mogelijkheid om een biobanken met biopten op te zetten en materiaal uit te wisselen.

Het gebruik van biopten heeft een meerwaarde voor onderzoek in vergelijking met de kweek van losse cellen, omdat in biopten de interacties tussen cellen onderling en met het omliggende weefsel behouden blijven. Echter, veel ziekteprocessen zoals een verdikking van het synovium kunnen in deze biopten niet meer worden onderzocht, omdat deze al plaatsgevonden hebben. Daarnaast is er grote variatie tussen de weefselstukjes die uit de biopten kunnen worden gehaald. Om de mogelijkheden voor onderzoek naar het synovium in een 3-dimensionale setting te verbeteren, hebben wij geprobeerd om een modelsysteem van een andere onderzoeksgroep verder te verbeteren in **Hoofdstuk 5**. Hiervoor hebben we gewrichtscellen losgemaakt en gemengd met Matrigel, dat vloeibaar is als het koud is, maar stolt bij 37°C. Gedurende de eerste 7-14 dagen in kweek bewogen de belangrijkste cellen uit het synovium, de fibroblast-achtige en macrofaag-achtige cellen, naar de oppervlakte en vormden een membraan dat veel lijkt op het synoviale membraan voordat de ziekteprocessen beginnen. Deze 3-dimensionale kweekmethode wordt micromass genoemd. Als we deze micromasses gedurende 3 weken stimuleerden met ontstekingsprikkel TNFα, dan ontstond een verdikking van het synovium, wat een bekend ziekteproces is. Daarnaast bleek dat de micromasses met het in hoofdstuk 4 beschreven protocol konden worden ingevroren, zonder verlies van de gevoeligheid voor ontstekingsprikkels. De micromasses kunnen dus een waardevol model zijn voor het bestuderen van nieuwe medicijnen op ziekteprocessen van het synovium.

Gentherapie met de lentivirale vector CXCL10p-IL10

De *Saa3* promotor die het best functioneerde in de muisstudie van hoofdstuk 2 bleek een stuk minder bruikbaar in menselijke cellen. De humane analoog van de *Saa3* promotor zorgt bijvoorbeeld niet voor de productie van een eiwit. Voor de vertaling van de IL-10 gentherapie naar de mens zijn we daarom op zoek gegaan naar een andere promotor in **Hoofdstuk 6**. Hiervoor is gekeken van welke eiwitten

de productie in een reumatisch gewricht verhoogd is in vergelijking met een gezond gewricht. Dit was het geval voor 22 eiwitten, waarvan de promotors dus kandidaten zijn voor ziektegestuurde gentherapie in menselijke cellen. Vervolgens is gekeken in gekweekte gewrichtscellen met een ontstekingsprikkel welk gen het meest geactiveerd werd en dit bleek *CXCL10* te zijn. De promotor van *CXCL10* is daarom gekopieerd naar een luciferase reporter. Deze bleek vervolgens sterk geactiveerd te raken door verschillende ontstekingsprikkels en door bloed van reumapatiënten. Bovendien nam de activiteit van de promotor na een ontstekingsprikkel in de loop van de tijd weer af, om vervolgens weer gevoelig te worden voor een nieuwe prikkel; een belangrijke eigenschap voor een ziektegestuurde therapie. Daarom is de luciferase reporter achter de *CXCL10* promotor vervangen door IL-10 tot de CXCL10p-IL10 lentivirale vector, de beoogde gentherapie voor de menselijke cellen. Deze therapie is in menselijke gewrichtscellen geplaatst en na een ontstekingsprikkel werd inderdaad een verhoogde productie van IL-10 gemeten in het kweekmedium. De geproduceerde hoeveelheid IL-10 bleek voldoende om tegelijkertijd de productie van ontstekingseiwitten zoals TNFα en IL-1β te remmen.

Na deze resultaten hebben we de CXCL10p-IL10 gentherapie getest in micromasses gemaakt van OA synovium in **Hoofdstuk 7**. Ook hier bleken ontstekingsprikkels TNFα en lipopolysaccharide (LPS) te zorgen voor een verhoogde productie van IL-10 door de micromasses. En ook in de micromasses vonden wij hierdoor een afname van de ontstekingsfactoren IL-1β en IL-6. De therapeutische kracht van de IL-10 geproduceerd met de *CXCL10* promotor was vergelijkbaar met die van de continu actieve *PGK* promotor. Daarnaast vonden we net zoals in de muisstudie van hoofdstuk 2 een verhoogde productie van het ontstekingsremmende eiwit SOCS3 na behandeling met IL-10. Hiermee hebben wij aangetoond dat onze CXCL10p-IL10 behandeling ook succesvol is in een geavanceerd model van menselijk synovium.

Eindconclusie

Concluderend hebben wij in dit proefschrift aangetoond dat ziektegevoelige gentherapie met het ontstekingsremmende eiwit IL-10 in staat is om de ontstekingsreactie te onderdrukken in zowel een muismodel van artritis, alsook in humane gewrichtscellen en in een 3-dimensionaal model van het humane synovium. Dit is een belangrijke stap in de zoektocht naar een medicijn voor reumatische ziekten dat voor langere tijd een gewricht kan beschermen, terwijl de blootstelling van andere organen wordt beperkt.

Curriculum Vitae

Martinus Gerardus Antonius Broeren werd geboren op 14 oktober 1987 in Venlo en groeide op in Arcen. Na het behalen van zijn Gymnasium diploma aan het Valuascollege in Venlo in 2006 begon hij met de studie Medische Biologie op de Radboud Universiteit. Tijdens de masterfase van zijn opleiding verrichte hij twee stages. In de eerste stage onderzocht hij de epigenetische achtergrond van X-chromosoom inactivatie op de afdeling Moleculaire Biologie, voor zijn laatste stage onderzocht hij onzuiverheden in het productieproces van insuline bij MSD in Oss.

Zijn diploma behaalde hij in januari 2012, waarna hij begon met zijn promotietraject op het laboratorium van de afdeling Reumatische Ziekten in het Radboudumc. Dit onderzoek onder leiding van professor Wim van den Berg en Fons van de Loo was gericht op het ontwikkelen van een ziekte-gevoelige gentherapie voor de behandeling van artritis. De resultaten van dat onderzoek staan beschreven in dit proefschrift. Sinds april 2016 richt hij zich op de verdere implementatie van het cryopreservatieprotocol dat is beschreven in hoofdstuk 4 van dit proefschrift en werkt hij onder leiding van Rogier Thurlings aan een methode om autoreactieve B cellen te isoleren uit het bloed van patiënten met het syndroom van Sjögren.

List of Publications

Broeren MG, Pieters BC, Bennink MB, Thurlings RM, Koenders MI, van der Kraan PM, van Lent PL, van den Berg WB, de Vries M, van de Loo FA. Micromasses of primary synovial cells reassemble into a TNF-alpha-responsive synovial membrane that can be viably cryopreserved. [Manuscript in preparation]

Broeren MG, Di Ceglie I, Bennink MB, van Lent PL, van den Berg WB, Koenders MI, Blaney-Davidson EN, van der Kraan PM, van de Loo FA. TSG-6 gene therapy in experimental osteoarthritis results in ectopic bone formation. [Submitted]

Broeren MG, de Vries M, Bennink MB, Arntz OJ, van Lent PL, van der Kraan PM, van den Berg WB, van den Hoogen FH, Koenders MI, van de Loo FA. Suppression of the inflammatory response by disease-inducible interleukin-10 gene therapy in a three-dimensional micromass model of the human synovial membrane. Arthritis Res Ther. 2016 Aug 12;18:186.

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** These authors contributed equally to this manuscript*

Dankwoord

Het schrijven van een proefschrift is niet mogelijk zonder de hulp van een hoop mensen. Niet alleen vanwege de wetenschappelijke ondersteuning, maar ook voor de prettige sfeer, waardoor ik mijn promotietraject in het warme nest van Lab Reuma als heel fijn heb ervaren. Ik wil dan ook iedereen bedanken die hieraan heeft bijgedragen, maar in het bijzonder de volgende mensen:

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Rogier, mijn tweede copromotor, als arts ben jij van ons degene die uiteindelijk het beste weet wat zich in de patiënt zelf afspeelt en die kennis is onmisbaar bij het onderzoek naar een ziekte. Daarnaast heb je mij de kans gegeven om na het aflopen van mijn aio-contract mijn kennis te verbreden naar de B cellen en het isoleren van die cellen uit het bloed van patiënten met een auto-immuunziekte. Tijdens onze (lange) ritjes naar het AMC voor (korte) meetings is er veel gelegenheid om te speculeren over moeilijke experimenten.

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Voor het behoud van een positieve energie en ontspanning zijn op sommige momenten de clubjes en verenigingen een heel goede uitkomst gebleken. Op zondagavond zijn er natuurlijk altijd trouw de heren van C.O.W.art: **Michiel**, **Bram**, **Jasper** en **Maik**. "Wat doen jullie dan? Wat voor muziek maken jullie?" Tja, we drinken koffie en daarna bedenken we een nummer in een genre dat we nog niet eerder gespeeld hebben. Wie het wil horen moet onze SoundCloud maar opzoeken. En Michiel, nog extra bedankt voor het maken van de kaft. Als ik had geweten dat je ook met de computer een mooie micromass kunt maken, dan had mij dat veel confocale microscopie kunnen besparen. Gedurende mijn promotie heb ik ook de eer gehad om hopman te mogen zijn van de verkenners van **Jong Arcen**. Ik vind het bijzonder dat we het elk jaar voor elkaar krijgen om voor alle groepen samen –van 6 tot 18 jaar– een onvergetelijk zomerkamp te organiseren en dat we onder het genot van wat ploppertjes uren kunnen kletsen bij het kampvuur. Ook mijn teamgenoten bij schaakvereniging **UVS**, met recht Uit Vrienden Samengesteld, zorgden voor een

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